USING STABLE ISOTOPES OF NITROGEN ($\delta^{15}N$) TO EXAMINE THE SOURCES AND PATHWAYS OF FOREST NITROGEN CYCLES: A GLOBAL META-ANALYSIS AND FIELD STUDIES IN ALASKAN BLACK SPRUCE FOREST

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To all the ecologically important yet grossly understudied fungi of the world and my lovely fiancée whom has weathered life in Florida during this foolhardy endeavor.

ACKNOWLEDGMENTS

I would first like to acknowledge the following people for assisting me at the University of Florida: my committee chair and advisor, E.A.G. “Ted” Schuur, for being supportive in my pursuit of an independent research track and for entertaining so many impetuous ideas; Grace Crummer for steadfast laboratory assistance and management through thick and thin; the infamous Jason and Cathy Curtis for assistance modifying the mass spectrometer - a feat that easily saved a year of my life; Andrea Albertin for assisting me with the adoption of the bacterial denitrifier technique; Hakån Wallander for discussing hyphal ingrowth bags; Erland Bååth for personally showing me PLFA extraction techniques in Lund, Sweden; Erik Hobbie, Ayato Kohzu, and Bernd Zeller for generously providing their previously published raw data on fungal isotopes; Paulo Brando and Reinhold Kliegl for providing statistical advice on mixed effect models; Jason Vogel and Juan Posada for early discussions on dissertation topics; and last but not least, Hollie Hall for providing unfailing support through it all.

With regards to work in Guyana, I would like to specifically acknowledge; the invaluable field assistance provided by the endearing Patamona Amerindians, Cathie Aime’s assistance with fungal identifications, Terry Henkel’s steadfast support, the Guyanese undergraduate researcher Clydecia McClure, and graduate and undergraduate students from Humboldt State University. Work in Guyana was made possible by: the Guyana Environmental Protection Agency research and export permits, funding from the Working Forests in the Tropics Graduate Research Award supported by the National Science Foundation (DGE-

0221599) to me, the National Geographic Society Research and Exploration Grant to Terry Henkel, and Mellon Foundation funds to Ted Schuur.

With regards to work in Alaska I would like to specifically acknowledge the expert field assistance by Martin Lavoie and Emily Tissier, as well as the countless hours of lab assistance back at UF by Dominique Ardura, Rady Ho, Dat Nyguen, and Rachel Rubin. I would also like to thank Steve Allison and Kathleen Treseder for providing sporocarp $\delta^{15}N$ data from the fertilized plots near Delta Junction. Work in Alaska was made possible by funding from the National Science Foundation Doctoral Dissertation Award (DGE-0221599), the Forest Fungal Ecology Research Award of the Mycological Society of America, the International Association of GeoChemistry Student Research Grant, the Riewald-Olowo University of Florida Graduate Research Award, and multiple UF Graduate Student Council Travel Awards to me; DOE and NSF funding to Ted Schuur; and, the support offered by the Ecosystem Ecology Laboratory of Terry Chapin and the Bonanza Creek Long Term Ecological Research site to Ted Schuur and Michelle Mack that supported the logistics of my research while living in Alaska.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the Requirements for the
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December 2010

Chair: Edward A.G. Schuur
Major: Botany

Ecosystem ecologists are challenged by both the scale and complexity of their discipline. Consistent methodologies and integrative metrics can ameliorate such challenges. Stable isotope ratios of nitrogen ($\delta^{15}N$) offer themselves as unique time-integrated proxies of numerous biophysical processes. The key N cycling information contained in ecosystem $\delta^{15}N$ values, however, is obscured by multiple competing hypotheses. Here, I examine patterns in $\delta^{15}N$ values in fungi from around the world and in multiple ecosystem components along the soil-fungi-plant continuum in boreal black spruce forest of central Alaska. My objective was to determine both the causes of underlying $\delta^{15}N$ variability and the utility of these measurements.

By combining previously published isotopic data with a novel dataset collected in poorly studied tropical rainforest, I was able to demonstrate the universal ability of dual isotope ($\delta^{15}N$ and $\delta^{13}C$) datasets to discriminate ectomycorrhizal from saprotrophic fungi in >90% of 813 samples of fungi. Furthermore, I demonstrated that the isotope values

could be partially predicted by climate in a manner similar to that previously demonstrated in plants and soils.

In Alaska I focused on severely N-limited forests dominated by a single species of ectomycorrhizal tree. By examining 31 plots varying widely in foliar δ^{15}N values, topography, and stand density, I aimed to understand the fundamental controls over δ^{15}N variability in plants. Using 16 experimentally fertilized plots I examined how changes to soil nutrient fertility can alter δ^{15}N values, N sources, and pathways of cycling. Using a highly sensitive bacterial denitrifier technique I overcame a methodological impasse of previous work and demonstrated that black spruce δ^{15}N values were a product of the interaction of soil fertility and ectomycorrhizal activity, not merely a reflection of source N δ^{15}N values. In the fertilized plots I demonstrated that only the combination of N and phosphorus fertilization leads to a significant reduction of black spruce reliance upon ectomycorrhizal-derived N from 82 to 46% of total N nutrition. Phosphorus fertilization, in particular, also led to an unusual and previously undocumented response in soil N cycling.

CHAPTER 1
INTRODUCTION AND OVERVIEW OF DISSERTATION

My PhD dissertation work has sought to push the boundaries of stable isotope applications in ecology and ecosystem science by defining the ecological roles of fungi and through examining the function of boreal ecosystems from the perspective of N cycling. An advantage of my approach is that it is multidisciplinary and multivariate. For instance, I have used advanced analytical and chemistry techniques to examine stable isotope ratios, microbial biomass, and elemental abundances in a diversity of organisms and soils. These techniques involved extensive methods development, instrument modifications, and visiting of foreign laboratories. I have also applied modern multivariate and information theoretic statistical techniques to address global and local hypotheses. Lastly, my work has involved taxonomic ID and physiological knowledge of fungi and plants in both tropical and boreal ecosystems.

Ecosystem ecologists are challenged by both the scale and complexity of their discipline. Because of difficulties in large-scale research, consistent methods and metrics are needed, and because of the complexity of ecosystem processes, integrators of space and time are needed. I chose to use stable N isotope ratios ($\delta^{15}N$) as a proxy metric of the form and pathway of the N cycle because it is naturally occurring, readily measurable, interacts strongly with biological organisms, and N is one of the primary mineral nutrients limiting ecosystem productivity.

There have been numerous publications detailing $\delta^{15}N$ patterns across a diverse range of organisms, soils, and environments. However, this body of literature, much of which is very recent, contains numerous examples where mechanistic interpretation of ecosystem $\delta^{15}N$ patterns is muddied by confounding underlying causes. A major

difficulty has been making replicate $\delta^{15}N$ measurements of bioavailable soil N pools at low field concentrations due to analytical limitations. Adoption of the bacterial denitrifier method at UF was therefore one of the major contributions of my PhD research as it allowed me to overcome this methodological impasse and test previously intractable hypotheses. Fundamentally, my research is an attempt at pinpointing specific causes of plant and fungal $\delta^{15}N$ variability so that ecologists may feel confident in using it as a time integrated proxy of the form and pathway of N cycling.

In **Chapter 2** I used a meta-analytical approach to assign ecological roles to numerous species of fungi collected from around the world. The isotopic differences among fungi have been described from single sites again and again but the universality of the pattern was not explored, particularly in tropical forests. By combining previously published isotopic data with a novel dataset collected in tropical rainforests of Guyana, I was able to demonstrate universal patterns of stable isotope values and relate this back to general ecological functions and climatic controls over biogeochemical cycles.

Why would an ecologist want to know the ecological roles of fungi? The simplest answer is because mycorrhizal and saprotrophic fungi exist at the biogeochemical interface between soils and plants. They are essential to terrestrial element cycling due to their uptake of mineral nutrients and decomposition of detritus. Therefore, understanding their ecology is vital to the understanding of ecosystem function – be it decomposition, soil respiration, or mineral nutrient cycling. Furthermore, ectomycorrhizal fungi in particular have been shown to fractionate heavily against the stable isotope of N, ^{15}N; demonstrating the universality of this is informative to future modeling efforts and predictive efforts regarding N cycling.

My approach toward these goals was to use a discriminant analysis of stable isotope ratios of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) from 813 fungi across 23 sites. I was able to verify collector based categorizations of individual sporocarps as either ectomycorrhizal (ECM) or saprotrophic (SAP) in >91% of the fungi, and provide probabilistic assignments for an additional 27 fungi of unknown ecological roles. As sites ranged from boreal tundra to tropical rainforest, I was able to show that fungal $\delta^{13}C$ and $\delta^{15}N$ values could be roughly predicted by climate or latitude mirroring what was previously shown in plant and soil analyses. These results are applicable to biogeochemists, evolutionary mycologists, and ecosystem ecologists. This chapter was published in *Ecology Letters* as a *Synthesis and Review Article* and has initiated several collaborative opportunities.

Chapter 3 continues my approach to understanding variability in, and applications of, $\delta^{15}N$, now in the context of a severely N limited boreal black spruce forest of central Alaska where the influence of ECM fungi on $\delta^{15}N$ patterns are expected to be particularly strong. The objectives of this work were to understand causes in black spruce $\delta^{15}N$ variability with the belief that the large variability observed was not simply due to variation in the source N, as hypothesized elsewhere, but rather dependency on ECM fungi for N nutrition. I chose 31 stands that varied widely in foliar $\delta^{15}N$ values, stand biomass, soil fertility, and topographic position with the goal of differentiating the underlying causes of ecosystem $\delta^{15}N$ variability across the range of black spruce growth conditions.

Multiple regressions were evaluated to explain variability in both elemental and biomass patterns of plants and fungi using the information theoretic approach of model

selection. Both foliar N and P contents were confirmed to reflect soil fertility; particularly soil dissolved organic N (DON) and resin exchangeable phosphorus. Black spruce foliar δ^{15}N values were best explained by a multidimensional soil fertility metric (principle component), either alone or in combination with the δ^{5}N values of soil N forms. Foliar and root δ^{15}N values covaried, as expected, yet the magnitude of the difference converged, under the least ^{15}N depleted conditions, calling into question many previous modeling assumptions that assumed a constant difference. Fungal sporocarp δ^{5}N values were negatively correlated with the DON content of soils, but not negatively correlated with black spruce δ^{15}N values, as theoretically expected. *In situ* measurements of fungal biomass increased with C:N ratios of organic soil reflected an interplay between soil N content and plant C allocation. Soil N δ^{15}N values, when incorporated into a series of mass balance isotope mixing models, indicated that black spruce is highly dependent on ECM derived N (86-98% of total N nutrition) despite variability in foliar δ^{15}N. Furthermore, in line with the predicted importance of organic N to boreal forest plants, DON was required to achieve mass balance in many of these mixing model solutions.

Chapter 4 extends the previous chapter's natural gradient approach to an experimental one. Notably, the same analytical approach was applied within a 5-year-old full factorial N and P fertilization experiment, also in central Alaskan black spruce forest. Because climate induced nutrient mineralization may increase N availability, experimental manipulation of soil nutrient availability offers insight into a possible trajectory of future conditions and evaluates the use of δ^{15}N as a proxy indicator of N cycling changes. Combining similar measurements from Ch. 3 with mass balance

mixing models it was shown that N+P fertilization lead to a 36% decline in N dependency on ECM forming fungi. Specifically, we found that N fertilization, both singly and in conjunction with P, caused the $\delta^{15}N$ values of foliage, fine roots, soil N, and fungal fruiting bodies to approach that of the fertilizer. Surprisingly, P fertilization also influenced the N cycle leading to a 60-fold increase of resin exchangeable soil NO_3^- pool and a $\delta^{15}N$ enrichment of 17‰ relative to the control. Fertilization of nitrifying bacteria followed by fractionation against ^{15}N during N volatilization could account for these findings. Combined, our experimental approach illustrated that measuring numerous ecosystem components, particularly source $\delta^{15}N$ values, is necessary to understand how enhanced soil fertility in boreal black spruce forest can be detected and how it may influence ECM dependencies and access to DON.

In conclusion, my PhD dissertation work has sought to push the boundaries of stable isotope applications to understand the ecological roles of fungi and the function of boreal ecosystems with regards to the productivity-limiting N cycle. My research is an attempt at pinpointing specific causes of plant and fungal $\delta^{15}N$ variability so that ecologists may feel confidence in using it as a time integrated proxy of the form and pathway of N cycling. This work has laid the foundation for my upcoming postdoctoral work seeking to do the same in tropical forest.

CHAPTER 2
ELUCIDATING THE NUTRITIONAL DYNAMICS OF FUNGI USING STABLE ISOTOPES

Abstract

Mycorrhizal and saprotrophic fungi are essential to terrestrial element cycling due to their uptake of mineral nutrients and decomposition of detritus. Linking these ecological roles to specific fungi is necessary to improve our understanding of global nutrient cycling, fungal ecophysiology, and forest ecology. Using discriminant analyses of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotope values from 813 fungi across 23 sites, we verified collector-based categorizations as either ectomycorrhizal (ECM) or saprotrophic (SAP) in >91% of the fungi, and provided probabilistic assignments for an additional 27 fungi of unknown ecological roles. For sites that ranged from boreal tundra to tropical rainforest, we were able to show that fungal $\delta^{13}C$ (26 sites) and $\delta^{15}N$ (32 sites) values could be predicted by climate or latitude as previously shown in plant and soil analyses. Fungal $\delta^{13}C$ values are likely reflecting differences in C-source between ECM and SAP fungi, whereas ^{15}N enrichment of ECM fungi relative to SAP fungi suggests that ECM fungi are consistently delivering ^{15}N depleted N to host trees across a range of ecosystem types.

Introduction

Fungi function at two fundamental biogeochemical interfaces between soil and plants. Decomposer fungi mineralize organic carbon (C) compounds in detritus and liberate mineral nutrients in the process, while mycorrhiza-forming fungi, as mutualistic root extensions, enhance mineral, and perhaps organic, nutrient uptake in exchange for plant photosynthate (Leake & Read 1997). Within the large guild of ectomycorrhizal (ECM) fungi there are potential, though rare, ‘cheaters’ (Egger & Hibbett 2004; Douglas

2008) and species with proteolytic capabilities that may blur these distinctions (Chen *et al.* 2001; Buée *et al.* 2007). However, dividing fungi into saprotrophic (SAP) and ECM functional groups has proven useful to biogeochemical and ecological research despite considerable variation among fungal species (Read & Perez-Moreno 2003; Gadd 2006). Ectomycorrhizal fungi are a diverse assemblage of ~7,000-10,000 spp. that mutualisticaly associate with woody plant hosts in a number of families of dominant tree species (*e.g.,* Pinaceae, Fagaceae, Dipterocarpaceae, Myrtaceae subfamily Leptospermoideae, and Fabaceae subfamily Caesalpiniodeae) in boreal, temperate, and to a more limited extent, tropical regions of the world (Halling 2001; Read & Perez-Moreno 2003; Taylor & Alexander 2005). The formation of macroscopic sporocarps (mushrooms) by many ECM and SAP fungi allows for experimental tractability unavailable for most microbial organisms. As a result, there are well developed species concepts, a rapidly developing comprehensive phylogeny (Hibbett *et al.* 2007), and unique opportunities for advancing ecological research on forest nutrient cycles, anthropogenic impacts, and fungal interactions with host plants (Wardle *et al.* 2004; Clemmensen *et al.* 2006; Hobbie & Hobbie 2006; Buée *et al.* 2007; Treseder *et al.* 2007).

Assigning ecological roles to individual taxa is necessary to conduct research on the biogeochemical importance of fungi. Assignment has typically been based on the following methods: 1) fruiting on, and presumed decomposition of, dead plant tissue by SAP fungi, 2) exclusive co-occurrence of sporocarps with ECM forming host plants, 3) phylogenetic distance to fungi with previously categorized ecological role, 4) direct tracing of hyphae from sporocarp to ECM rootlet, 5) molecular comparison of sporocarp

to ECM rootlet, and 6) dual isotope values of C and N to determine nutritional mode. However, each of these methods has limitations: 1) fungal growth on well decomposed wood and/or aerial fruiting habits can confound categorization based solely on fruiting substratum (Henkel *et al.* 2006); 2) exclusive co-occurrence of sporocarps with suitable host plants is often unresolved due to inadequate field observation; 3) the evolutionary 'switching' by many ECM forming basidiomycete fungi makes assignment to ecological role based on phylogeny alone questionable (Hibbett *et al.* 2000; Matheny *et al.* 2006); 4) direct evidence, such as tracing hyphae from fruiting body to ECM rootlet, is difficult or impossible to obtain in most soil matrices; and, 5) while molecular comparisons certainly can link ECM root tips to sporocarps (Horton & Bruns 2001), their widespread adoption by ecologists remains technologically and financially constrained. In this study, we explored the ability of the isotope based method to assign ecological roles to fungi by quantifying the error associated with the technique.

The ^{15}N:^{14}N and ^{13}C:^{12}C stable isotope ratios (expressed as $\delta^{15}N$ and $\delta^{13}C$ in permil (‰) values relative to standards) of fungi provide time integrated biogeochemical information regarding the acquisition, transformation, and export of C and N by fungi under natural conditions (Griffith 2004). Unique C and N cycling pathways in ECM and SAP fungi lead to different isotope fractionation effects (Hobbie & Wallander 2006). The $\delta^{15}N$ enrichment of ECM relative to SAP fungi is thought to result from assimilation and transfer of ^{15}N depleted N to host plants, a process that cumulatively leads to fungal enrichment and host plant depletion (Hobbie *et al.* 1999; Högberg *et al.* 1999a; Hobbie *et al.* 2005). Thus, the absence of N transference to plants by SAP fungi causes them to appear ^{15}N depleted relative to ECM fungi within a site. Patterns of $\delta^{13}C$ in fungi are

largely attributed to isotope differences in the substrate(s) used as an energy source. Saprotrophic fungi use C from plant tissues and soil organic compounds comprised of diverse C sources each with distinct $\delta^{13}C$ values often 1-6 ‰ different from more commonly measured bulk plant foliage or roots (Gleixner *et al.* 1993; Marshal *et al.* 2007; Bowling *et al.* 2008), whereas ECM fungi receive plant photosynthate C that is isotopically more homogeneous relative to plant tissue (Gleixner *et al.* 1993; Högberg *et al.* 1999b; Henn & Chapela 2001; Baldocchi & Bowling 2003). The resulting differences in either fungal $\delta^{15}N$ or $\delta^{13}C$ have been used to differentiate ECM from SAP fungi (Taylor *et al.* 1997; Gebauer & Taylor 1999; Hobbie *et al.* 1999; Högberg *et al.* 1999b). Recently, the simultaneous use of both isotopes has improved this approach (Kohzu *et al.* 1999; Hobbie *et al.* 2001b; Taylor *et al.* 2003; Trudell *et al.* 2004; Clemmensen *et al.* 2006; Hart *et al.* 2006; Zeller *et al.* 2007). However, global variability in C and N isotope values in plants and soils (Amundson *et al.* 2003) suggests that cross site comparisons may only be possible following some form of site normalization to correct for differences in average isotopic baselines among sites (Henn & Chapela 2001; Post 2002). Isotopic baselines are the $\delta^{15}N$ or $\delta^{13}C$ values of basal C and N sources within a trophic system or food web, such as photosynthate or labile mineral nutrient sources (reviewed in Post 2002). If large-scale site variability in baseline isotope patterns is partially attributed to climate, such as with plant and soil $\delta^{15}N$ and $\delta^{13}C$ values (Amundson *et al.* 2003; Craine *et al.* 2009), then site normalization of $\delta^{15}N$ or $\delta^{13}C$ values would clarify the influence of physiology and nutrition on ECM and SAP C and N isotope patterns.

The accumulation of multiple datasets from around the northern hemisphere has enabled us to address both the utility and cause of C and N isotope differences in ECM

and SAP fungi. In order to determine if fungal $\delta^{15}N$ or $\delta^{13}C$ patterns across ecosystems are similar to plants and soils, we assessed the explanatory capacity of mean annual temperature (MAT), mean annual precipitation (MAP), and latitude (LAT). Cross site comparisons were optimized by use of site based normalization to remove variability associated with changes in background isotope values and uneven ECM and SAP sampling within sites. We then used a large number of published and unpublished fungal $\delta^{15}N$ or $\delta^{13}C$ values to statistically discriminate ecological categorizations (SAP vs. ECM) of fungi with suspected and unknown ecological roles. Isotope values in fungi provide a form of ecological information independent of phylogenetics, soil excavation, or molecular sequencing, and when combined with one or more of these other techniques, provides definitive evidence of the nutritional ecology of specific fungi and can inform biogeochemical and evolutionary research in many of the world's forested ecosystems.

Methods

Data Assembly

To test global predictions of ECM and SAP isotope patterns, we compiled $\delta^{15}N$ and $\delta^{13}C$ values from one novel and ten published data sets. Compiled data included 913 $\delta^{15}N$ and 813 $\delta^{13}C$ values from collector categorized ECM or SAP fungi, and 27 fungi of unknown ecological role, together comprising 148 genera. Of the 32 study sites included, 30 were from temperate, boreal, subarctic, or arctic ecosystems and two from tropical rainforest (site descriptions available from references listed in Table 1). The tropical sites included fungi from a dipterocarp dominated Malaysian rainforest (Kohzu *et al.* 1999) and a Guyanese rainforest dominated by a leguminous tree. Lowland

tropical rainforest sites with ECM trees, while underrepresented in our analysis, are globally uncommon outside of dipterocarp or caesalpinioid dominated rainforests as well (Taylor & Alexander 2005). Fungal $\delta^{15}N$ data from areas with high levels of atmospheric N deposition from anthropogenic sources were excluded to eliminate possible confounding of natural fungal $\delta^{15}N$ patterns (Gebauer & Taylor 1999; Lilleskov *et al.* 2002b). For each site, MAT, MAP, and LAT were compiled from original manuscripts, or extrapolated from nearby climate stations when not reported.

Guyana Field Site and Sample Processing

Fungal sporocarps were collected during the 2003-2006 June-August rainy seasons in the Upper Potaro River Basin in the Pakaraima Mountains of Guyana (5°18'04.8"N, 59°54'40.4"W; elevation 710 m). The moist evergreen forests in this region receive 3855 mm yr^{-1} of rain, and occur on well drained, highly oligotrophic soils that are low in phosphorus (P), calcium, and magnesium, and high in iron and aluminum (Henkel 2003; Mayor & Henkel 2006). The fresh foliar N:P mass ratio of 25.5 (N = 5 canopy sun leaves) for *Dicymbe corymbosa* Spruce ex. Benth (Caesalpiniaceae) and the highly weathered parent material are suggestive of P limiting conditions to primary productivity (Güsewell 2004).

Fungal sporocarps were collected from monodominant stands of the ECM forming canopy tree, *D. corymbosa*, morphologically described while fresh, identified to species or morphospecies, and field-dried with desiccants (Henkel *et al.* 2002). Additional herbarium specimens collected previously from this area within the last 10 years were also analyzed for isotope values to target taxonomically unusual fungi of unknown ecological role. In the laboratory, equal portions of pilei and stipe or entire sporocarps

from 56 fungi were finely ground, dried at 60˚C for 24 hours, and analyzed on a ThermoFinnigan continuous flow isotope ratio mass spectrometer coupled to a Costech elemental analyzer at the University of Florida. Stable isotope abundances are reported as: $\delta^{15}N$ or $\delta^{13}C = (R_{sample}/R_{standard} - 1) \bullet 1000$, where R = $^{15}N/^{14}N$ or $^{13}C/^{12}C$ of the sample and reference standard (atmospheric N_2 and PeeDee belemnite-C, respectively). Run error rates were typically ≤0.2‰ for $\delta^{15}N$ and ≤0.1‰ for $\delta^{13}C$. Voucher specimens for Guyana fungi are maintained at Humboldt State University (HSU).

Data Analyses

Linear regressions were conducted on site mean $\delta^{15}N$ and $\delta^{13}C$ values to assess the individual ability of MAT, MAP, and LAT to explain ECM and SAP mean isotope patterns. Best fit polynomial equations (linear versus second order) were chosen based on R^2 comparisons. Combined, 9 fungi from Tanigawa and Okinawa, Japan (Kohzu *et al.* 1999) were removed from $\delta^{15}N$ correlations as extreme statistical outliers indicated by box-plots. The presence of significant correlations among site mean fungal $\delta^{13}C$ and predictor variables indicated that accuracy in discriminant categorization of ECM and SAP fungi could indeed be enhanced through a normalization procedure as previously suggested (Henn & Chapela 2001).

To normalize datasets prior to discriminant analysis, we separately calculated the mean $\delta^{15}N$ and $\delta^{13}C$ values of the ECM and SAP groups within each site. Next, we averaged these two group means and subtracted this new unbiased mean from each individual fungal isotope value. This normalization procedure removed the overall sampling bias toward ECM fungi across sites (62% were ECM) and centered site

means to zero. Site normalization excluded isotope values from four fungi representing sites with only single ecological categories (Kagoshima, Okinawa, and Oodai, Japan). A comparable, yet more complicated site normalization procedure was previously used (Henn & Chapela 2001) on a subset of the data presented here. Their site normalization procedure was designed to examine the contributions of fungal physiology and ecology (e.g. substrate) on ^{13}C fractionation in fungi. Because of this objective, their site corrections involved 'removal of the difference in means between ECM and SAP fungi for C data' (Henn & Chapela 2001) to correct for substrate and highlight remaining isotopic differences caused by physiology. Our relatively more simple transformation procedure was designed to retain the ECM-SAP differences, regardless of cause, while reducing cross-site variability indicated by the abiotic proxies of climate and latitude. Therefore, our normalization procedure was applied to both C and N isotope values without the assumptions needed for substrate corrections.

We used standard discriminant multivariate analysis of both site normalized and actual fungal isotope values to: 1) statistically test for a global isotopic difference among ECM and SAP ($\Delta_{ECM-SAP}$) fungi, 2) assign collector based categorization error terms for fungi, and 3) categorize fungi of unknown ecological role from several of the sites using probabilities arising from the entire dataset. In the discriminant analysis, probabilities of categorical assignment were set proportional to occurrence, and because the assumption of equivalent covariance among variables was not met, a pooled variance quadratic function was used instead.

Discriminant analyses have been described as circular processes because they use predefined groups to inform categorization of those same groups (Quinn & Keough

2005). To alleviate this concern we validated categorization with a second discriminant analysis using a 50% random subset of the data to categorize all remaining fungi, and a separate cluster analysis using only fungal isotope values to assign groups (Quinn & Keough 2005). The specific categorical assignments of individual sporocarps were compared among the original and subsequent analyses to test for consistency in sporocarp categorization. The above analyses were conducted using JMP® 7.0.2 (SAS Institute Inc.).

Following site normalization and discriminant analyses we sought to more fully examine the combined ability of MAT, MAP, and LAT, to explain fungal $\delta^{13}C$ and $\delta^{15}N$ patterns using linear mixed effect models. Mixed effect models were necessary because both fungal species and isotope values within sites are non-independent clustered observations, and therefore violate a parametric test requirement of uncorrelated error terms associated with each measurement (Faraway 2006). Modeling non-independent variables as random effects is a well established statistical technique (Crawley 2007; Andersen 2008) that allowed each sample point in our dataset, as opposed to just site means as in linear regression, to inform linear model construction.

Mixed effect models were constructed using the *lme4* (Bates 2008) and *lattice* (Sarkar 2008) packages in R® (version 2.6.2; R-Development Core Team 2008). All models had the dependent variables of sporocarp $\delta^{13}C$ and $\delta^{15}N$ described by ecological group (ECM or SAP), site, and either species or genus, both with and without nested interactions. In addition, MAT, MAP, and LAT, were compared in a factorial fashion as additive, multiplicative, or quadratic predictor variables to determine the most informative formulation. These complex forms were justified by the possibility of

nonlinear and conditional interactions among predictors as indicated by visual assessment of scatter plot matrices. Centering of MAT, MAP, and LAT (subtraction of each value from overall mean) optimized model fitting procedures and, consequently, reduced multicolinearity among predictor variables. Beginning with the most complex model, mathematical formulations of MAT, MAP, and LAT were sequentially compared using maximum likelihood methods. Following convergence on the optimal fixed effect configuration (e.g. lowest Akaike information criterion (AIC) value), we evaluated random effects, with either species or genera nested within site, using restricted maximum likelihood methods (Crawley 2007). We assessed model quality using the common graphical diagnostics of residuals against fitted values, sample quantile against theoretical quantile plots, and regressions of predictor variables for interpretation of effects (Quinn & Keough 2005). AIC was used to select final models because it is widely regarded as an unbiased estimator that assess relative model fit, penalizes over parameterization, and allows multiple working hypotheses to be simultaneously evaluated (Andersen 2008).

Results

Dual $\delta^{13}C$ and $\delta^{15}N$ graphs of fungal sporocarps illustrate a global divide in isotope values between different ecological categories of fungi (Figure 2-1). On average, ECM fungi were significantly ^{15}N enriched and ^{13}C depleted relative to SAP fungi (5.5 ±0.16 vs. -0.3 ±0.16‰, and -25.5 ±0.06 vs. -23.0 ±0.09‰, ±SE; $P < 0.001$, t-test). Global ranges in sporocarp $\delta^{15}N$ (-7.1 to 21.8‰) and $\delta^{13}C$ (-31.7 to -19.0‰) values were broad and the range of MAT (-8.5 to 26 ˚C) and MAP (300 to 3866 mm yr^{-1}) among sites represents much of global climatic variability in terrestrial ecosystems (Table 2-1).

Sorting fungi into either tropical (N = 2) or extra-tropical (N = 30) sites indicated that fungi from the tropical sites were significantly more ^{13}C depleted and ^{15}N enriched than extra-tropical sites (SAP: $P < 0.05$, two-tailed t-test; ECM: $P < 0.05$, Wilcoxon rank sum test). Despite absolute differences among site mean $\delta^{13}C$ and $\delta^{15}N$, the magnitude of isotopic differences between ECM and SAP fungi was consistent across sites (Table 2-1) and not significantly different from a slope of one (Figure 2-2; $\alpha < 0.05$).

The large number of sporocarp dual isotope measurements used in the discriminant analysis (N = 813) allowed for reliable assignment of collector based categorization error (Appendix A-2), and consequently, confident categorization of fungi of unknown ecological role based upon >50% statistical probabilities (Appendix B-2). Collector based categorizations using normalized isotope values were deemed valid in 91.2% of sporocarps indicating a high level of agreement with and among categorization methods used by collectors. Site normalization reduced collector categorization error by 0.7% relative to non-normalized isotope values, and retained within site variability. Normalization appears to have only slightly reduced overlap between ECM and SAP groups (Figure 2-1B) due to centering of all site means to zero. The increased accuracy of discriminant categorization could be due to increased model efficiency during extraction of eigenvector distances (Quinn & Keough 2005).

Discriminant categorization of fungi was further supported by the additional discriminant analysis of a random 50% subset of the data, and cluster analysis of the entire dataset. The random 50% subset increased overall discriminant categorization error by only 0.3% and was in agreement with individual categorizations based on the entire dataset. The cluster analysis also identified ecological categories but increased

overall categorization error by an additional 1.5%. These values are similar to error rates derived from the discriminant analysis using the full data set and are indicative of low *a priori* categorical-forcing of ecological groups due to categorical assignment by collectors. Combined, these results suggest that sporocarp categorization is robust to a substantial reduction in data input and that collector knowledge, while accurate, is of little statistical importance in predicting fungal ecology relative to $\delta^{13}C$ and $\delta^{15}N$ values alone.

Climate and latitude serve as reasonably good predictors of fungal isotope values on a global scale. Linear regression of site mean ECM and SAP fungal $\delta^{13}C$ indicated significant relationships with MAT (R^2_{adj} = 0.18 and 0.63, respectively) and LAT (R^2_{adj} = 0.49 and 0.67, respectively), but not with MAP, whereas $\delta^{15}N$ was significantly correlated with MAT (R^2_{adj} = 0.24) in ECM fungi only (Figure 2-3). Singly, MAP and LAT had no predictive power over mean fungal $\delta^{15}N$, but the inclusion of MAT, MAP, and LAT as centered quadratic variables in linear mixed models substantially improved their explanatory power (i.e. $\Delta_I > 2$; Table 2-2). Removal of LAT from mixed models was deemed useful because it produced comparable model fits as MAT and MAP and reduced obvious correlations among predictor variables. Remaining multicolinearity among centered MAT and MAP was low (R^2 = 0.13) and both variables were retained despite potential similarity in information. Linear mixed models indicated that variability in sporocarp $\delta^{13}C$ was best explained by MAT, LAT, an interaction between them (MAT*LAT), and sporocarp type (ECM vs. SAP; Table 2-2). Substituting the random effect of genus for species also substantially increased model fit (Δ_i = 71.2; Table 2-2). Use of genus allowed for more informative mixed models due to the presence of the

same fungal genera across multiple sites, while species were often confined to single locations. Sporocarp $\delta^{15}N$ was best described by the fixed effects MAT, MAP, and their quadratic functions (Table 2-2). Altering random effects to include the interaction of species with site improved model fit, and, as with $\delta^{13}C$, substituting the random effect of genus for species produced a substantially more informative model ($\Delta_i = 83.0$; Table 2-2).

Discussion

Implications of the Global Pattern

The $\delta^{13}C$ and $\delta^{15}N$ based division in ECM and SAP fungi has been described from localized sites and is shown here to exhibit a consistent pattern across the global range of ecosystem types. The magnitude of isotopic differences between ECM and SAP fungi was also similar across sites, as evidenced by a slope similar to one (Figure 2-2), and parallel relationships of site mean $\delta^{13}C$ and $\delta^{15}N$ values with climate and latitude (Figure 2-3). Site normalization eliminated correlations with climate and latitude but reduced discriminant categorization error by only 0.7%. The similar isotopic differences suggest comparable ecophysiological functioning of these two nutritional groups of fungi across sites that differ widely in climate, plant community, and baseline isotope values. Because of the ubiquity of the pattern, the assembled dataset can be used to confidently (>90%) categorize the nutritional status of fungi based on $\delta^{13}C$ and $\delta^{15}N$ values of sporocarps or hyphae (Wallander *et al.* 2004). Furthermore, site normalization and collector knowledge increased prediction accuracy, but only by a small margin.

The observed ^{15}N enrichment of ECM relative to SAP sporocarps is attributed to ^{15}N discrimination (Δ8–10‰) during the formation and delivery of amino acid N from fungi to plants (Hobbie & Hobbie 2006). Additional causes of ECM ^{15}N enrichment relative to SAP fungi could include preferential use of ^{15}N enriched forms of N as well as internal processing within the fungus irrespective of transfer to the host plants (Hobbie & Colpaert 2003; Brearley *et al.* 2005; Dijkstra *et al.* 2008). However, field and laboratory observations currently support the N delivery to host plant process as being most influential in terms of isotope fractionation. In addition, frequently observed ^{15}N depletion in ECM associating host plants relative to co-occurring non ECM plants further supports this hypothesis (Hobbie & Hobbie 2008). Lower $\delta^{15}N$ values in non-ECM forming plants are expected because the assimilation and transfer of N by arbuscular mycorrhizal (AM) fungi is thought to impart a smaller ^{15}N fractionation (Δ0–2‰) in host plants (Handley *et al.* 1993; Craine *et al.* 2009). Therefore, a globally similar ECM-SAP divide suggests that ECM fungi are delivering ^{15}N depleted N to host plants under both N limitation to plant productivity, such as might be found in temperate and high latitude ecosystems, as well as in tropical forests that could be limited primarily by P (Palmiotto *et al.* 2004; Mayor & Henkel 2006). In support of these expectations, a recent analysis of foliar $\delta^{15}N$ from 91 studies used the type of root symbiosis (ECM and ericoid mycorrhizal vs. AM or non-mycorrhizal) to explain 29% of the variation in foliar $\delta^{15}N$ globally (Craine *et al.* 2009).

Because ECM and SAP fungi differ fundamentally in C source (i.e. plant photosynthate vs. detritus), sporocarp $\delta^{13}C$ is expected to track the $\delta^{13}C$ values of these two major C pools (Högberg *et al.* 1999b; Henn & Chapela 2001). Although the $\delta^{13}C$

values of fungi are thought to reflect patterns found in plant C pools, they are typically 0.3-5‰ more enriched than host tissue (Gleixner *et al.* 1993; Hobbie *et al.* 1999; Högberg *et al.* 1999b; Hobbie *et al.* 2001b; Trudell *et al.* 2004; Kohzu *et al.* 2005; Hart *et al.* 2006). This offset from known (and suspected) substrates may be due to ^{13}C discrimination during decomposition by SAP fungi or during synthesis and translocation of various C pools from host plants to ECM fungi (Bowling *et al.* 2008; Högberg *et al.* 2008). Saprotrophic fungi tend to be relatively more ^{13}C enriched (relative to bulk leaves) than either ECM fungi and their plant sugar C source, or leaf lipids and proteins (Bowling *et al.* 2008). This differential offset from presumed C source suggests additional fractionation pathways may be contributing to SAP ^{13}C enrichment in particular (Kohzu *et al.* 2005). Regardless of physiological contributions to fungal $\delta^{13}C$, differences in the $\delta^{13}C$ values of C pools are considered responsible for the ^{13}C divide among ECM and SAP fungi.

Our analysis illustrates that it is possible to reliably infer ecological roles of fungi over a broad range of conditions using a relatively simple, site based normalization procedure of fungal $\delta^{13}C$ and $\delta^{15}N$ values. It has been suggested that the ECM-SAP divide should become less distinct over large geographical areas and that the isotopic signature of an organism is insufficient evidence to infer ecological role without first standardizing to an appropriate isotopic baseline (Henn & Chapela 2001; Hobbie *et al.* 2001b; Post 2002; Taylor *et al.* 2003). However, our normalization slightly enhanced the resolution of the discriminant analysis through removal of mean isotopic differences among sites without comparison to a measured baseline. Given the small decrease in discriminant categorization error following site normalization (0.7%) it is unlikely that

correcting to isotopic baseline measurements (leaves and mineral nutrients) could reduce categorization error further. For instance, isotopic corrections based on foliar samples could introduce variation based on sample position in the canopy, taxonomic grouping, growth form, or type of root symbiosis (Pate & Arthur 1998; Bustamante *et al.* 2004; Craine *et al.* 2009). Additionally, isotopic corrections based on soil $\delta^{15}N$ could introduce variation depending on depth, disturbance, and fertility of the samples (Bustamante *et al.* 2004; Davidson *et al.* 2007; Dijkstra *et al.* 2008).

In addition to the discrimination of fungal ecological roles across multiple ecosystems, our dataset shows an ECM-SAP difference ($\Delta_{ECM\text{-}SAP}$) in $\delta^{15}N$ of 5.3‰ and -2.3‰ for $\delta^{13}C$; values that will help constrain the magnitude of isotope fractionations used in modeling efforts. Fractionation attributed to ECM mediated N assimilation and transfer to host plants were recently used to estimate that Alaskan tundra plants received 61-86% of their total N from ECM fungi and reciprocally delivered 8-17% of their photosynthetic C to ECM fungi (Hobbie & Hobbie 2006). These simultaneous mass balance calculations require accurate assessment of the fractionation magnitude associated with ECM delivered N in order to constrain additional estimated, and interdependent, variables (Hobbie & Hobbie 2008). This quantification of elemental cycling is a necessary step to the modeling of N cycles and the partitioning of below ground C allocation. Values in our global analysis can refine this approach because additional fractionation effects, such as during N uptake, have been partially removed through the subtraction of co-occurring SAP $\delta^{15}N$ values. Better parameterization of this key ^{15}N fractionation step is necessary for expansion to, and testing of, C and N mixing models in additional ecosystems (Hobbie & Wallander 2006).

Climatic Influence on Sporocarp $\delta^{15}N$

Fungal $\delta^{15}N$ patterns were expected to correlate with MAT, MAP, and LAT to the extent that soil and litter $\delta^{15}N$ values correlate with those values. In previous global isotopic analyses, MAT and MAP were found to be good predictors of soil and plant $\delta^{15}N$ due to their influence over soil N cycling and the isotope ratios of ecosystem N inputs and outputs (Amundson *et al.* 2003; Craine *et al.* 2009). This is because warm temperature and high rainfall conditions are conducive to high rates of N mineralization and nitrification leading to a loss of ^{15}N depleted N through denitrification or leaching and consequent ^{15}N enrichment of soil N (Amundson *et al.* 2003; Templer *et al.* 2007). Therefore, the integrated N pool in tropical soils is typically ^{15}N enriched relative to high latitude ecosystems with low soil N availability and conservative N cycles (Högberg 1997; Amundson *et al.* 2003). Significant sporocarp $\delta^{15}N$ enrichment was observed in fungi from tropical relative to temperate sites but the predictive ability of individual variables to explain fungal $\delta^{15}N$ patterns was only weakly correlated with MAT. The curved relationship among ECM $\delta^{15}N$ values and MAT (Figure 2-3B) was driven by the coldest tussock tundra site near Toolik, Alaska (Clemmensen *et al.* 2006), the exclusion of which removed the relationship. Causes for the higher than expected $\delta^{15}N$ values in ECM sporocarps at Toolik, AK (12‰) could result from large proportional N transfer by ECM fungi to severely N limited plants (Hobbie & Hobbie 2006) or ECM and SAP access to anomalously ^{15}N enriched N sources (Lilleskov *et al.* 2002b). Whereas individual variables were poor predictors of fungal $\delta^{15}N$, enhanced explanatory power in mixed effect models illustrate that MAT and MAP do hold predictive ability when properly formulated ($\Delta_i = 9.9$; Table 2-2). In summary, the expectation that fungal $\delta^{15}N$

patterns would respond similarly to that seen for plants and soils was not supported suggesting that fungal physiology exerts primary influence over fungal $\delta^{15}N$ patterns. However, the inclusion of fungal and foliar $\delta^{15}N$ values from more tropical and subtropical sites will undoubtedly improve the predictive ability of discriminant and mixed models and help clarify the secondary influence of climate on sporocarp $\delta^{15}N$.

Climatic Influence on Sporocarp $\delta^{13}C$

Mean $\delta^{13}C$ values from fungi in the warm/wet sites were most similar to those from the cold/dry sites when viewed in relation to MAT and LAT as single predictor variables. As with $\delta^{15}N$, we determined the influence of the coldest site (Toolik Lake, AK) by removing it, however in this case the relationship between fungal $\delta^{13}C$ and MAT remained (R^2_{adj} = 0.16 ECM, 0.56 SAP). Plant analyses of foliar $\delta^{13}C$ generally provide reliable indices of plant water use efficiency (WUE) owing to ^{13}C discrimination during photosynthesis and its relation to stress induced stomatal closure (Marshal *et al.* 2007). However, the weak relationship between fungal $\delta^{13}C$ and MAP in our analysis indicated that this coarse climatic variable was of little utility in explaining fungal, and presumably plant, C isotope patterns across such diverse sites. For instance, the four driest sites in our meta-analysis differed widely in temperature (-8.5 to 5˚C), as did the four wettest (9.5 to 25˚C). Therefore, partitioning individual climatic influences over fungal $\delta^{13}C$ at these extreme conditions is confounded by the joint possibility of greater water stress at dry sites and temperature induced photosynthetic inhibition at the coldest ones similarly altering plant, and indirectly fungal, $\delta^{13}C$ (Allen & Ort 2001). Regional analyses show MAP gradients are strongly correlated (R^2 = 0.64 to 0.70) with foliar $\delta^{13}C$ in Northern and Southeastern Australia, respectively (Stewart *et al.* 1995; Austin & Sala 1999), but

not Hawaii (Schuur & Matson 2001). It is more likely that measures of actual or potential evapotranspiration would be more informative in future studies.

Latitude explained the largest portion of mean fungal $\delta^{13}C$ variability among sites (R^2_{adj} = 0.49, ECM; 0.67, SAP) likely due to its integration of multiple effects on plant $\delta^{13}C$ patterns, and combined with MAT and their interaction, substantially increased the explanatory power of mixed models (Δ_i = 26.8; Table 2-2). The proxies of MAT and LAT likely integrate the timing and form (snow vs. water) of precipitation among sites during growing seasons, as well as other physiological stressors that can modify plant WUE and $\delta^{13}C$ values, such as: 1) within species physiological variability (Pate & Arthur 1998; Schuur & Matson 2001; Kohzu *et al.* 2005); 2) species replacement, particularly at low soil water contents (Swap *et al.* 2004); 3) relative N availability and C sink strength of ECM fungi (Hobbie & Colpaert 2003); and 4) compensatory effects of specific leaf area and leaf N concentration on plant WUE (Schulze *et al.* 2006). Similarly, a global meta-analysis of 1,248 plants across 452 sites demonstrated the ability of MAT and LAT to explain patterns in foliar N and P at global scales, suggesting a reflection of both plant physiological adjustments and the relative shifts in nutrient limitations due to changes in the age of soils (Reich & Oleksyn 2004).

Predictions of Fungal Ecology

Despite the strength of the discriminant analyses, known errors in model categorization have been found in our dataset suggesting other, unknown errors are likely. Known 'SAP categorization errors' are confirmed wood decay fungi that were categorized as ECM by the discriminant model with >90% probability. These apparent errors included the following individual sporocarp collections: *Pleurotus ostreatus*

(Jacq.) P. Kumm. from Chiba, Japan*, Microporus vernicipes* (Berk.) Kunt. from Lambir, Malaysia (Kohzu *et al.* 1999); and *Gymnopilus bellulus* (Peck) Murrill, from Lamar Haines, Arizona (Hart *et al.* 2006). Including other apparent SAP errors with <90% modeled probabilities increases the SAP errors by six individual samples (Appendix A-2). Error in the categorization of decomposer fungi could be partially caused by unique nutritional sources in addition to wood or litter. For instance, access to recent plant photosynthate with relatively low $\delta^{13}C$, or to accruing microbial and insect biomass with relatively high $\delta^{15}N$, could cause SAP fungi to 'appear' as ECM in the discriminant model.

Discriminant model errors regarding ECM fungi are more difficult to discern because of the difficulty in determining the nutrition of terrestrial sporocarps. Several individual sporocarps belonging to genera and species traditionally categorized as ECM were categorized as SAP by the discriminant model with >90% probability. These presumed 'ECM categorization errors' included: *Cortinarius sp.* (Pers.) Gray Kyoto, Japan, *Tylopilus sp.* P. Karst. from Lambir, Malaysia (Kohzu *et al.* 1999); *Cortinarius variosimilis* M.M. Moser & Ammirati, from Deer Park Rd, WA (Trudell *et al.* 2004); and *Cantherellus pleurotoides* T.W. Henkel, Aime and S.L. Mill. from Guyana (this study). Including other, typically ECM genera (*e.g., Amanita, Russula, Lactarius, Boletus*) with lower than 90% probabilities would increase this number by 23 individual samples (Appendix A-2). Causes for these discrepancies are unknown but warrant caution in accepting predictions based on dual isotope measures of single sporocarp collections in the absence of other evidence (Trudell *et al.* 2004). Triplicate analyses of six species in our Guyana dataset, collected across years, had standard deviations of 0.61 to 0.71

($\delta^{13}C$ and $\delta^{15}N$, respectively) and were all assigned to the same ecological group by the discriminant model. When anomalous isotope values do occur for individual sporocarps across sites, particular caution of categorization error is warranted. At low sampling number, site normalized values are likely to be less accurate (e.g. not a true site mean) due to insufficient sporocarp numbers. This is particularly problematic if using archived specimens from distant or poorly documented sites. In contrast, when the same fungal species is categorized 'incorrectly' across multiple sites or collections it may offer insight into unique nutritional, kinetic, or physiological activities that run counter to traditional predictions in groups otherwise considered to have a narrow nutritional mode.

Classification of fungi with unknown ecological roles using site normalized dual isotope measurements produced confident predictions (i.e. probability >90%) for 19 out of 27 fungi in our dataset, and weak to moderate predictions (50.2 to 89%) for the remaining eight (Appendix B-2). Such predictions could form the basis for research hypotheses about the evolution of the ECM and SAP habit (Hobbie *et al.* 2001b) and be combined in a phylogenetic context (Wilson *et al.* 2007). For instance, the inclusion of *Phylloporus rhodoxanthus (*Schwein.) Bres., a lamellate genus included within an otherwise poroid hymeniphore forming family (Boletaceae), adds ecological support to the micro-morphological, molecular, and observational data categorizing this genus as ECM. In addition, the previously uncertain ecological roles of *Clavulina* Schroet in Cohn, *Helvella* L., *Coltriciella* Murrill, and *Tremellodendron* G.F. Atk., now have strong isotopic evidence for the ECM mode of nutrition; and in conjunction with phylogenetic affinity to other presumed ECM taxa (Henkel *et al.* 2005), now have additional support (Appendix B-2).

In agreement with natural history based methods, accurate identification of fungal genera is useful for predicting the ecological role of many fungi. Consistent generic categorization in the discriminant model and improved linear mixed model predictive capacity using genus support this. The observation that $\delta^{15}N$ patterns of fungal genera can exhibit either high or low $\delta^{15}N$ (Trudell *et al.* 2004) and $\delta^{13}C$ syndromes (Kohzu *et al.* 1999) suggest that future studies may seek to define a stable isotope based niche space similar to the metrics used to describe tidal food webs (Layman *et al.* 2007). It is intriguing to speculate that specialization of some fungal genera with depth (Lindahl *et al.* 2007; Taylor *et al.* 2010), N source (Lilleskov *et al.* 2002b; Hobbie & Hobbie 2008), or other niche dimensions could be represented by an index of sporocarp $\delta^{15}N$ and $\delta^{13}C$ variability. If demonstrated, this could also contribute to how genus increased the ability of mixed models to predict fungal $\delta^{15}N$ and $\delta^{13}C$ patterns globally.

Summary

The C and N isotopic difference between ECM and SAP fungi provides researchers with a reliable tool for the ecological categorization of fungi regardless of site origin. This time integrated, biogeochemical evidence offers insight into C and N cycles across most forested ecosystems and highlights the global importance of ECM fungi to host plant N nutrition and forest N cycling. The ecophysiology associated with ECM and SAP nutritional roles was shown to exert primary control over fungal $\delta^{13}C$ and $\delta^{15}N$ values, however some of the variability was attributable to climatic and latitudinal proxies. These findings offer a tool by which fungi can be integrated into our understanding of global elemental cycles and illustrates that fungal $\delta^{15}N$ may be partially decoupled from soil and plant isotope patterns. As evidenced here, datasets containing

fungi from tropical forests contributed to the reliability of stable isotope analyses to discriminate ecological roles of any sporocarp producing fungus and the future inclusion of fungi from subtropical and southern hemisphere sites will undoubtedly improve our confidence in this approach.

Table 2-1. Summary of isotopic, climatic, and ecological data by study and site.

		(*N*)			(˚C)	(mm yr^{-1})	ECM (‰)		SAP (‰)		$\Delta_{ECM-SAP}$	
Ref.*	Site	ECM	SAP	LAT	MAT	MAP	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
1	Aheden, Sweden	53	0	64	1.0	600	N/A	6.0	N/A	N/A	N/A	N/A
	Betsele, Sweden	5	0	64	1.0	570	N/A	8.1	N/A	N/A	N/A	N/A
	Flakaliden, Sweden	21	0	64	2.3	600	N/A	6.1	N/A	N/A	N/A	N/A
	Kulbacksliden, Sweden	8	0	64	1.2	523	N/A	4.3	N/A	N/A	N/A	N/A
	Norrliden, Sweden	1	0	64	1.6	595	N/A	5.4	N/A	N/A	N/A	N/A
	Svartberget, Sweden	5	0	64	1.6	595	N/A	5.0	N/A	N/A	N/A	N/A
	Vilan, Sweden	7	0	64	5.1	542	N/A	5.3	N/A	N/A	N/A	N/A
						Study mean	--	*5.7*	--	--	--	--
2	Aishu, Japan	19	21	35	11.7	2353	-24.6	5.0	-22.6	1.6	-2.0	3.5
	Chiba, Japan	3	6	35	14.7	1550	-26.2	-0.8	-23.7	-0.6	-2.6	-0.1
	Kagoshima, Japan	0	1	31	17.8	2236	N/A	N/A	-22.9	-7.1	N/A	N/A
	Kyoto, Japan	28	26	35	15.8	1814	-24.6	4.8	-23.1	0.6	-1.5	4.2
	Lambir, Sarawak Malaysia	17	14	4	26.0	2700	-26.8	7.6	-25.0	-0.4	-1.8	7.9
	Miyajima, Japan	1	1	34	17.0	1546	-24.6	9.8	-22.1	1.5	-2.5	8.3
	Norikura, Japan	9	3	36	6.7	2766	-24.3	8.4	-21.6	-0.1	-2.7	8.5
	Okinawa, Japan	1	0	24	23.0	1736	-25.0	21.2	N/A	N/A	N/A	N/A
	Ontake, Japan	9	8	35	6.7	2766	-24.1	2.4	-21.8	-2.7	-2.3	5.1
	Oodai, Japan	0	2	34	15.7	1511	N/A	N/A	-22.2	-0.2	N/A	N/A
	Shirahama, Japan	1	2	33	16.8	1730	-24.5	4.4	-23.1	0.7	-1.5	3.8
	Tanigawa, Japan	2	6	36	5.2	1692	-24.8	19.1	-22.3	-1.0	-2.5	20.0
						Study mean	*-25*	*8.2*	*-22.7*	*-0.7*	*-2.1*	*6.8*
3†	Glacier Bay Alaska, USA	4	4	59	14.9	1830	-25.4	4.5	-22.9	-1.9	-2.5	6.4
4	Mixed conifer California, USA	18	25	N/A	N/A	N/A	-25.8	9.0	-22.5	-0.1	-3.3	9.1
5	Woods Creek Oregon, USA	20	25	45	11.0	1000	-26.2	3.9	-22.8	-1.8	-3.5	5.7

Table 2-1. Continued

Ref.*	Site	(*N*) ECM	(*N*) SAP	LAT	(˚C) MAT	(mm yr^{-1}) MAP	ECM (‰) $\delta^{13}C$	ECM (‰) $\delta^{15}N$	SAP (‰) $\delta^{13}C$	SAP (‰) $\delta^{15}N$	$\Delta_{ECM-SAP}$ ECM	$\Delta_{ECM-SAP}$ SAP
6	Aheden, Sweden	29	4	64	1.0	600	-25.8	7.8	-23.3	-0.5	-2.4	8.4
	Stadsskogen, Sweden	110	13	59	5.5	541	-25.7	5.8	-23.1	1.6	-2.6	4.2
						Study mean	*-25.8*	*6.8*	*-23.2*	*0.5*	*-2.6*	*6.3*
7	Deer Park Rd Washington, USA	64	23	47	9.0	1150	-25.4	5.5	-23.3	-1.2	-2.1	6.7
	Hoh River Washington, USA	54	38	47	10.0	3450	-25.2	4.7	-22.9	-2.3	-2.3	7.0
						Study mean	*-25.3*	*5.1*	*-23.1*	*-1.8*	*-2.2*	*6.9*
8	Snowbowl Arizona, USA	9	13	35	4.0	775	-24.0	4.6	-22.0	1.9	-2.0	2.7
	Lamar Haines Arizona, USA	12	13	35	5.0	775	-24.0	3.2	-21.9	2.4	-2.1	0.8
						Study mean	*24.0*	*3.9*	*-22.0*	*2.2*	*-2.0*	*1.7*
9	Heath tundra, Sweden	10	4	68	-1.0	300	-27.0	1.7	-23.7	0.08	-3.4	1.7
	Tussock tundra Alaska, USA	3	5	68	-8.5	350	-26.4	12.0	-24.7	3.0	1.8	9.0
						Study mean	*-26.7*	*6.9*	*-24.2*	*1.6*	*-2.5*	*5.3*
10	Breuil-Chenue, France	33	14	47	9.0	1280	-26.2	3.1	-22.8	-2.8	-3.5	5.9
	Spruce plantation, France	20	17	47	9.0	1280	-24.1	3.7	-22.6	-0.6	-1.4	4.3
						Study mean	*-25.1*	*3.4*	*-22.7*	*-1.7*	*-2.5*	*5.1*
11	Upper Potaro River, Guyana	29	20	5	24.0	3866	-26.0	5.7	-24.9	1.6	-1.1	4.1
	Sum	*605*	*308*			*Grand mean*	*-25.3*	*6.4*	*-22.9*	*-0.3*	*-2.3*	*5.3‡*

Number (*N*) of individual ectomycorrhizal (ECM) and saprotrophic (SAP) sporocarps collected from each site. The mean annual temperature (MAT) and precipitation (MAP) values correspond to either published or extrapolated measurements from nearby climate stations. Differences in mean isotope values ($\delta^{13}C$ and $\delta^{15}N$) from each site were subtracted from one another ($\Delta_{ECM-SAP}$) to illustrate variability in ECM-SAP isotope differences among sites.

* References: 1, Taylor *et al*. 1997; 2, Kohzu *et al*. 1999; 3, Hobbie *et al.* 1999; 4, Henn & Chapela 2001; 5, Hobbie *et al.* 2001; 6, Taylor *et al.* 2003; 7, Trudell *et al.* 2003; 8, Hart *et al*. 2006; 9, Clemmensen *et al.* 2006; 10, Zeller *et al*. 2007; 11, this study.

† Values reported as generic means of 67 ECM and 29 SAP species.

‡ Tanigawa, Japan omitted as a statistical outlier.

Table 2-2. Competing linear mixed model results.

Model	*K*	Log-like	AIC	Δ_i*
$\delta^{13}C$				
Species				
Null	3	-1247.2	2504.3	102.4
MAT_c+LAT_c	5	-1246.2	2506.4	104.5
$MAT_c+LAT_c+(MAT_c+LAT_c)^2$	4	-1239.9	2491.7	89.8
$MAT_c:LAT_c$	4	-1237.1	2486.2	84.3
$(MAT_c+MAT_c^2)+(LAT_c+LAT_c^2)$	5	-1234.5	2485.0	83.1
$MAT_c+LAT_c+MAT_c:LAT_c$	6	-1228.5	2473.1	71.2
Genus				
Null	3	-1209.3	2428.7	26.8
$MAT_c+LAT_c+MAT_c:LAT_c$	6	-1193.0	2401.9	0.0
$\delta^{15}N$				
Species				
Null	3	-2245.1	4500.1	2088.4
$MAT_c+MAT_c^2+LAT_c+LAT_c^2$	7	-2241.6	4497.3	2085.7
$MAT_c+MAT_c^2+MAP_c+MAP_c^2$	7	-2241.1	4496.2	2084.6
Site:Species				
$MAT_c+MAT_c^2+MAP_c+MAP_c^2$ +SPECIES+SITE:SPECIES	9	-1275.9	2565.7	154.1
$MAT_c+MAT_c^2+MAP_c+MAP_c^2$ +SITE+SPECIES+SITE:SPECIES	10	-1239.3	2494.6	83.0
Genus				
Null+SITE:GENUS	6	-1204.8	2421.5	9.9
$MAT_c+MAT_c^2+MAP_c+MAP_c^2$ +SITE+GENUS+SITE:GENUS	10	-1196.4	2411.6	0.0

Models of $\delta^{13}C$ and $\delta^{15}N$ values in ectomycorrhizal (ECM) and saprotrophic (SAP) fungi were based on 913 $\delta^{15}N$ and 813 $\delta^{13}C$ fungal sporocarp measurements. The null mixed model contained the fixed effect of sporocarp type (ECM or SAP) and the random effects of site and either species or genus as indicated. Number of model parameters (*K*), log-likelihood (log-like), Aikake information criteria (AIC) model selection results, centered mean annual temperature (MAT_c), centered mean annual precipitation (MAP_c), centered absolute latitude (LAT_c).

* $\Delta_I = AIC_i - AIC_{min}$, where AIC_{min} is the minimum of the different AIC_i values and represents the information lost using other models with higher AIC scores. As a rule of thumb, an $\Delta_I \leq 2$ have substantial support for being more informative over competing models (Anderson 2008).

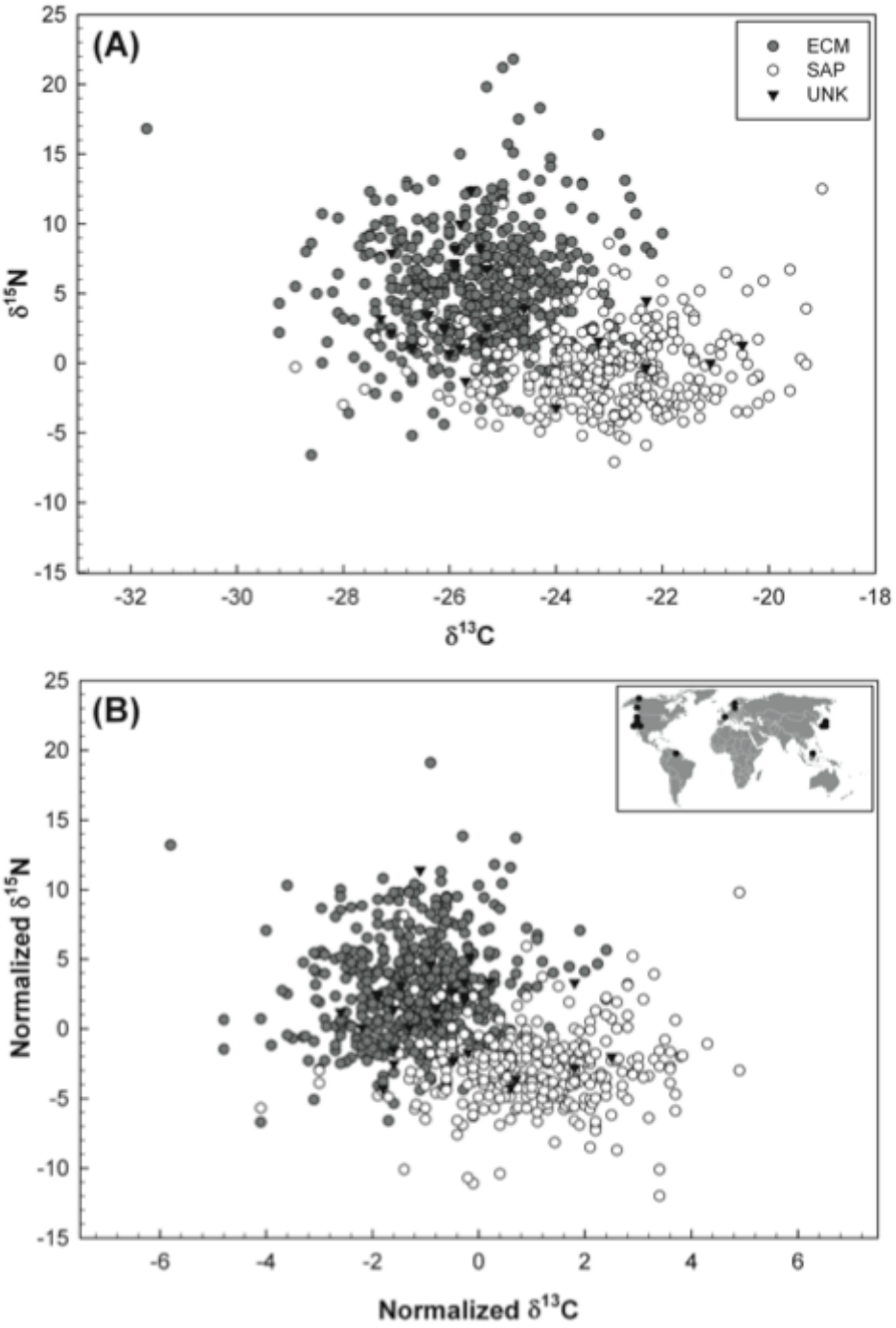

Figure 2-1. Dual isotope graphs of ectomycorrhizal (ECM), saprotrophic (SAP), and fungi of unknown (UNK) ecological role collected from 32 sites around the world. A) Raw isotope values. B) Site-normalized isotope values with inset illustrating the approximate site locations of sporocarp collections.

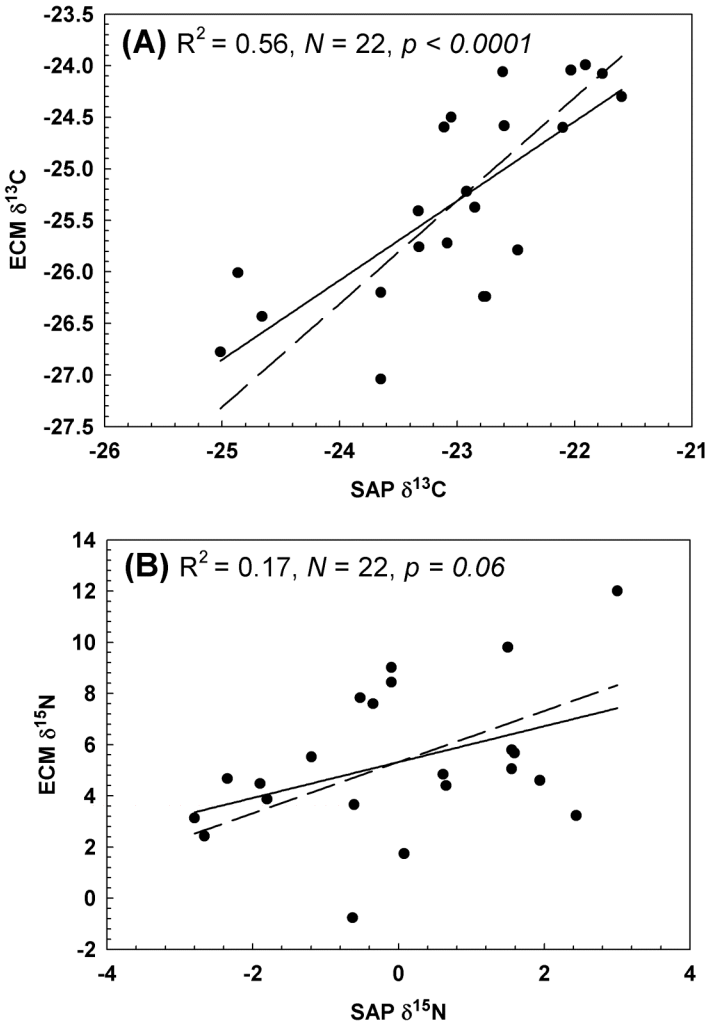

Figure 2-2. Comparison of the mean $\delta^{13}C$ and $\delta^{15}N$ of ectomycorrhizal (ECM) and saprotrophic (SAP) fungi from each site. A) $\delta^{13}C$ values. B) $\delta^{15}N$ values. Both A and B linear fits (solid line) are not significantly different from a slope of 1 (dashed line; $\alpha<0.05$) indicating a consistent isotopic difference between ECM and SAP fungi across sites. Tanigawa, Japan, was omitted as a statistical outlier. Equations of lines: (A) linear: f = -7.64 + 0.77χ, slope constrained: f = -2.32 + 1χ; (B) linear: f = -5.32 + 0.7χ, slope constrained: f = 5.32 + 1χ.

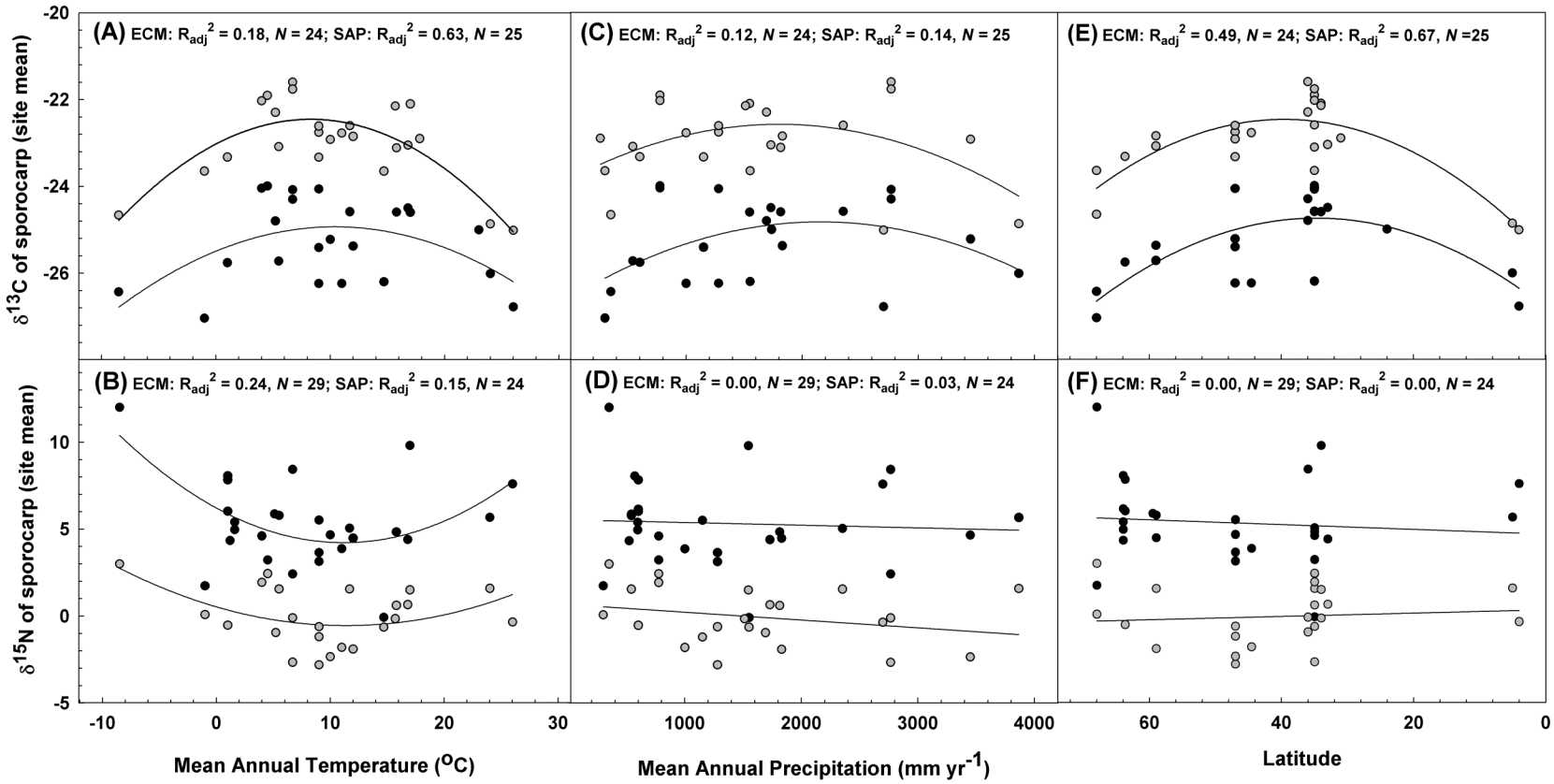

Figure 2-3. Regressions of site mean fungal $\delta^{13}C$ and $\delta^{15}N$ values with mean annual temperature, mean annual precipitation, and latitude. Black circles are site means of ectomycorrhizal (ECM) fungi; grey circles are site means of saprotrophic (SAP) fungi. (A) $\delta^{13}C$ values of fungi in relation to MAT. (B) $\delta^{15}N$ values of fungi in relation to MAT. (C) $\delta^{13}C$ values of fungi in relation to MAP. (D) $\delta^{15}N$ values of fungi in relation to MAP. (E) $\delta^{13}C$ values of fungi in relation to latitude. (F) $\delta^{15}N$ values of fungi in relation to latitude. Linear vs. quadratic best-fit polynomial equations were compared and equations producing the best R^2 coefficient are presented. Tanigawa and Okinawa, Japan, sites were removed from graphs B, D, F as statistical outliers.

CHAPTER 3
SOURCE VS. PATHWAY: $\delta^{15}N$ PATTERNS IN CENTRAL ALASKAN BLACK SPRUCE FOREST REFLECT HIGH DEPENDENCY ON ECTOMYCORRHIZAL-DERIVED ORGANIC NITROGEN

Abstract

The productivity of many northern ecosystems is nitrogen-limited but details of the N cycle remains little known. Recent analyses of global patterns in soil, plant, and fungal stable isotopes of N ($\delta^{15}N$) show promise in providing an integrative metric of N cycling processes, but differentiating the underlying causes of $\delta^{15}N$ variability remains a research priority.

We conducted a landscape-scale regression and modeling analysis of elemental patterns along the soil-fungi-plant continuum in 31 stands of central Alaskan black spruce. Because the stands varied widely in $\delta^{15}N$ values, biomass, soil fertility, and topographic position, we encompassed much of the variety of low-fertility black spruce stands and were able to specifically examine if fertility, soil N forms, or ^{15}N fractionation by ectomycorrhizal (ECM) fungi contributed to ecosystem $\delta^{15}N$ patterns.

Black spruce foliar $\delta^{15}N$ values were partially influenced by soil fertility and the $\delta^{15}N$ value of N sources whereas %N was most influenced by soil dissolved organic N (DON) content. Incorporation of ^{15}N fractionation by ECM fungi into isotope mixing models indicated that black spruce is heavily dependent on ECM derived N (86-98% of total N nutrition), much of which is acquired from the DON pool. These findings indicate that the elemental content of black spruce needles integrates both the source and pathway of N cycling as influenced by soil fertility and ECM fungal activity.

Introduction

The productivity and dynamics of many ecosystems are governed by nitrogen (N) availability; added N typically increases the biosynthesis of proteins and enzymes for photosynthesis (Galloway *et al.* 2004; Howarth & Marino 2006). Productivity of terrestrial ecosystems also typically increases in response to increased N availability (Vitousek & Howarth 1991; Elser *et al.* 2007). Because many human activities can increase N availability through deposition, or increase N-mineralization rates through climate warming and landscape modification, monitoring ecosystem responses to shifts in N availability is a research priority (Vitousek 1997; Mellilo & Cowling 2002). In N-limited boreal forests, slight changes in N availability can alter ecosystem productivity, carbon storage, and diversity of plants and microbes (Helfield & Naiman 2002; Högberg *et al.* 2003; Toljander *et al.* 2006; Treseder *et al.* 2007). Assessing ecosystem N supplies, flux pathways, and overall budgets, however, remains difficult and error prone even with frequent measurements because N cycling processes vary in both time and space (Binkley *et al.* 2000; Schimel & Bennett 2004; Cleveland *et al.* 2010).

Recent syntheses suggest that stable isotopes of N provide integrative measures of N cycling that can be used to infer N cycling processes at local, regional, and even global scales (Amundson *et al.* 2003; Pardo *et al.* 2006; Craine *et al.* 2009; Pardo & Nadelhoffer 2010). For instance, global $\delta^{15}N$ patterns in soils, fungi, and plants partially vary with mean annual temperature and precipitation (Handley *et al.* 1999; Amundson *et al.* 2003; Mayor *et al.* 2009). These large-scale patterns are thought to be due to the indirect control of climate over microbial activity, the “openness” of the N cycle, and the form of N lost from ecosystems (Gebauer & Schulze 1991; Nadelhoffer & Fry 1994; Högberg 1997; Martinelli *et al.* 1999; Schuur & Matson 2001; Pardo *et al.* 2006; Houlton

et al. 2007). At smaller scales, foliar $\delta^{15}N$ is linked to multiple processes in the N cycle including: the total amount and distribution of N within a plant; the N concentration, depth, and retention capacity of soils; and the relative rates of N-mineralization and uptake (Nadelhoffer *et al.* 1996; Falkengren-Grerup *et al.* 2004; Houlton *et al.* 2007; Templer *et al.* 2007). For instance, high soil N-mineralization rates are often correlated with greater losses of N by denitrification and leaching (Houlton *et al.* 2006; Kahmen *et al.* 2008). This loss of N forms (e.g. NO_3^-, N_2O, N_2) that are ^{15}N-light relative to their source pools causes ^{15}N enrichment of remaining soil N (Piccolo *et al.* 1994; Boeckx *et al.* 2005; Pörtl *et al.* 2007).

Because soil N sources can exhibit variable $\delta^{15}N$ values depending on relative amounts of ^{15}N-discrimination during ammonification, nitrification, and denitrification (Létolle 1980; Mariotti *et al.* 1981; Shearer & Kohl 1986), foliar $\delta^{15}N$ values are often thought to reflect specialization on specific N forms or rooting depths (Schulze *et al.* 1994; Michelsen *et al.* 1996; McKane *et al.* 2002; Houlton *et al.* 2007). Occasionally confounding this interpretation is the observation that the presence of ecto- and ericoid mycorrhizal root symbionts can greatly modify foliar $\delta^{15}N$ values from source to autotrophic sink during the production of ^{15}N depleted N transfer compounds (Michelsen *et al.* 1998; Högberg *et al.* 1999a; Hobbie & Hobbie 2008). This source of fractionation was highlighted globally in a regression model of >9,000 trees where 29% of global variability in foliar $\delta^{15}N$ values was attributed to mycorrhizal fractionation (Craine *et al.* 2009). Interestingly, the corresponding ^{15}N-enrichment of many mycorrhizal fungi is thought to contribute to the common pattern of ^{15}N-enrichment with soil depth (Lindahl

et al. 2007; Dijkstra *et al.* 2008; Etcheverría *et al.* 2009; Hobbie & Ouimette 2009; Wallander *et al.* 2009).

Despite the many insights about N cycling based on ecosystem $\delta^{15}N$ measurements, it remains difficult to differentiate the ultimate causes of plant $\delta^{15}N$ values because or multiple mechanistic possibilities (Craine *et al.* 2009; Pardo & Nadelhoffer 2010). For instance, the same plant $\delta^{15}N$ patterns could be caused by increasing lability of N, dependency on different forms of soil N, or dependence on differing mycorrhizal types. Addressing these simultaneous possibilities has proved methodological intractable because of the difficulty involved with making replicate $\delta^{15}N$ measurement of available soil N sources at field concentrations. Such detailed and high-throughput methods are now available and have been used in a diversity of terrestrial ecosystems (Ostle *et al.* 1999; Yoneyama & Tanaka 1999; Koba *et al.* 2003; Houlton *et al.* 2006; Houlton *et al.* 2007; Pörtl *et al.* 2007; Takebayashi *et al.* 2010; Yano *et al.* 2010). Continued methodological refinements will undoubtedly refine understanding of the terrestrial N cycle.

The objective of this research was to examine the underlying $\delta^{15}N$ variability among soil N pools (DON, NH_4^+, NO_3^-), soil fertility, and fungal biomass within the black spruce ecosystem of central Alaska. By measuring $\delta^{15}N$ values of source N moieties, modeling soil fertility parameters, and integrating isotope effects associated with ECM activity, we sought to evaluate the influence of both *source* and *pathway* on the N cycle along major pools in the soil-fungi-plant continuum. If plants simply trace soil N sources then black spruce $\delta^{15}N$ values should be well correlated with soil N $\delta^{15}N$ values even if ECM-based fractionation was taken into account. In contrast, if ECM fungi respond to

their autotrophic host's mineral nutrient requirements, then soil fertility should modulate the effected ECM-based fractionation in relation to the total proportion of black spruce N derived from them. A corollary of this prediction would then be that ECM biomass and activity should decline where soil fertility is greatest.

Black spruce forests are ideal systems in which to disentangle causes of $\delta^{15}N$ variability because they form monodominant stands over a broad range of N-limiting growth conditions ranging from low productivity stands on shallow permafrost soils to high productivity stands on well drained deep soils (Viereck & Johnston 1990; Chapin III *et al.* 2006; Hollingsworth *et al.* 2006; Ping *et al.* 2010). Because approximately 65% of Alaska is covered by boreal forests of which black spruce forest is the most abundant type covering approximately 40 million ha (Vancleve & Dyrness 1983), extrapolation of our findings to a larger geographical area will inform the greater boreal forest research community. Furthermore, because the N cycle is closely coupled to C sequestration, providing baseline data and interpretation of ecosystem $\delta^{15}N$ patterns can inform global change research as well.

Methods

Experimental Design

During 2007, 31 mature black spruce plots in central Alaska were selected and sampled from a pool of 146, previously established, 1 ha circular plots (Hollingsworth *et al.* 2006; Hollingsworth *et al.* 2008). The selected plots encompass an area approximately 14,000 km^2 and were selected based on the criteria that they were unburned or cleared, and represented the full range of foliar $\delta^{15}N$ values previously observed from analyses of 90 of the 146 plots (M.C. Mack, unpublished data).

Sampling in the plots occurred along a 30 m belt transect centered on a permanent plot marker and arrayed perpendicular to the slope or arbitrarily if flat. Cation exchange capacity (CEC), pH, stand diameter at breast height (dbh), and active layer depth for these sites were obtained using standard methods previously detailed (Hollingsworth *et al.* 2006). Black spruce biomass was estimated using updated equations derived from approximately 15 sites spanning the geographic range of or stands (Yarie *et al.* 2007). Black spruce fine roots were confirmed to be uniformly colonized by ECM fungi (Ruess *et al.* 2003) during root sampling in each stand.

Field and Laboratory Analyses

Foliar $\delta^{15}N$, %N, and %P, were obtained from mature black spruce terminal branches at the peak of needle expansion during August and September, 2007, to reduce variability associated with changing concentrations during needle expansion (Chapin & Kedrowski 1983) although a small portion of terminal needles represented previous years' growth. Five full sun branches were collected from five trees in each plot, composited by tree, and stored at 4^{o}C for less than 48 hours until drying. Preliminary analyses indicated that only three trees need be analyzed to encompass within plot variability. Small diameter (<2 mm) terminal root samples were also excavated from the same trees sampled for foliage and stored at 4^{o}C for approximately two weeks before processing. The thin outer layer of secondary root tissue was removed in order to prevent inclusion of external ECM biomass in subsequent elemental and isotopic analyses (Högberg *et al.* 1996) although some internal hyphae may have been present in the cortical tissue remaining. Both needles and roots were dried at 60^{o}C for 24 hrs, ground to a fine powder, and analyzed on a ThermoFinnigan® continuous flow isotope ratio mass spectrometer coupled to a Costech® elemental

analyzer at the University of Florida (UF). Stable isotope abundances are reported as: $\delta^{15}N = (R_{sample} / R_{standard} - 1) \times 1000$, where $R = {}^{15}N:{}^{14}N$ ratio of the sample or atmospheric N_2 standard. Run error rates were less than 0.2‰ as compared to NIST standards.

To compare relative plant available soil NO_3^-, NH_4^+, and PO_4^+ concentrations among plots, ion exchange resin beads were incubated throughout the entire 2007 growing season in accordance with methods detailed elsewhere (Giblin *et al.* 1994; Schuur & Matson 2001). During June 9-21, five replicate nylon mesh bags containing either 3 g of anion (Biorad®, AG 1-X8, 500g, 20-50 mesh, Cl- form, #140-1421) or cation exchange resins (Biorad®, AG 50W-X8, 500g, 20-50 mesh, H+ form, #142-1421) were inserted below sub-fibric organic soils at depths of 5-20 cm depending on the depth of undecomposed surface mosses. This placement was chosen to match the area of highest root density of black spruce forest observed in minirhizotrons (Ruess *et al.* 2003). Each of the five paired cation and anion resin bags were placed approximately 20 cm apart in equidistant locations along a 30 m belt transect. Resin bags were constructed of 220 µm polyester mesh bags (6.25 in^2), acid washed, and triple D.I. rinsed prior to deployment for the growing season.

In addition, five hyphal ingrowth mesh bags were inserted along the opposite side of the belt transect immediately above the organic-mineral interface to measure actively growing fungal mycelia (Wallander *et al.* 2001; Nilsson *et al.* 2004). The ingrowth bags were constructed of 52 µm nylon mesh bags containing an average of 7 cm^3 of acid washed triple D.I. rinsed moist quartz sand. Three additional bags were inserted inside buried PVC collars in each plot to account for saprotrophic fungal

biomass, but because negligible biomass was found within collared bags corrective accounting was unnecessary. Both resin and hyphal ingrowth bags were removed from the field at the end of the growing season in early September just prior to the first frost, refrigerated, and transported to UF for processing. Resin bags were extracted with 100 mL of an acidified salt (Giblin *et al.* 1994) and extracts frozen prior to colorimetric ion analysis on an Astoria® auto-analyzer. Hyphal ingrowth bags were slowly dried, suspended in a petri dish with water and carefully scored under a dissecting microscope for degree of colonization (1 = no hyphae, 2 = light and diffuse hyphae, 3 = extensive hyphae, 4 = extensive hyphae and light rhizomorphs, 5 = extensive hyphae and extensive rhizomorphs). Visual examination has previously been shown to correlate with weight and biochemical proxies of fungal biomass (Nilsson *et al.* 2004).

To obtain total dissolved nitrogen (TDN) samples, 2*M* KCl soil extractions were made at the height of the growing season (mid-July 2007). Three cores were extracted with an SMS® volumetric slide hammer (4.2 cm diameter) and composited from four zones along each 30 m transect in each plot (12 total cores per plot, 4 field replicates). The cores were located within 2 m of resin or hyphal ingrowth bag positions equidistant along belt transects. The depths of the organic, mineral, and total core were recorded, green moss removed, and the horizons bagged separately. Only organic soils were used in isotopic analyses due to the logistic limitations posed by the large number of samples. Total volumes extracted from each plot averaged nearly 1 m^3 of wet soil. Each composited soil sample was stored at 4^oC for ~24 hrs prior to homogenization and extraction. TDN was extracted from 24 g (wet weight) subsamples placed in a pre-cleaned and acid washed 250 mL HDPE plastic cup with 120 mL of 2*M* KCl in nano-

pure (Barnstead Thermo Scientific®) water. For each new batch of 2*M* KCl prepared, four 60 mL blanks were taken to correct for ^{15}N contamination associated batch specific impurities (Knapp *et al.* 2005). Soil and extractants were shaken on a reciprocal table for 20 minutes and left undisturbed for 18 to 30 hours. The resulting supernatant was vacuum filtrated through glass fiber filter papers (Whatman® 1820-070) in 9 cm HDPE Buchner funnels in the Ecosystem Ecology Laboratory at University of Alaska, Fairbanks. Extracts were frozen in replicate 50 mL centrifuge tubes and shipped to the UF for subsequent isotopic analyses during the following year.

Replicate 6 mL subsets of the salt extracted TDN and NH_4^+ ions were oxidized to NO_3^- using persulfate/thermo digestion (Cabrera & Beare 1993; Doyle *et al.* 2004) with an oxidant to sample ratio of 1:1.5. For each oxidation, standard solutions of 1, 5, and 10 mg of KNO_3^-, glycine, and 6-Aminocaproic acid (ACA) were used to assess digestion efficiency. It was found that digestion efficiency of 10 mg/L of ACA, the largest molecular weight and recalcitrant compound (MW = 131.17) at the highest concentration was improved to >87% at 0.5*M* salinity (versus 2*M* NaCl or KCl) presumably due to the interference of Cl^- with free radical generation (Peyton 1993). Accordingly, all samples were diluted to 0.5*M* with nano-pure water prior to oxidation. Following oxidation, $\delta^{15}N$ values from the resultant NO_3^- were measured using the highly sensitive bacterial denitrifier technique (Sigman *et al.* 2001; Knapp *et al.* 2005). This method involves: culturing of a naturally occurring denitrifying bacterial strain (*P. aureofasciens*); injection of small (~0.5 µg N) amounts of NO_3^- under anaerobic conditions; an overnight incubation period; then in-line cryo-purification of the resulting gaseous N_2O for isotopic analyses. All samples were analyzed on a gas sampler arm

coupled to a ThermoFinnigan® continuous flow isotope ratio mass spectrometer at UF. Extracted dissolved organic N (DON) was calculated using a mass weighted equation based on the original 2*M* KCl concentration of ions ([N]) and resin extracted NH_4^+ and NO_3^- isotope values:

$$\delta^{15}\text{N-DON} = (\delta^{15}\text{N-TDN} \times [\text{TDN}] - (\delta^{15}\text{N-NH}_4^+ \times [\text{NH}_4^+] + \delta^{15}\text{N-NO}_3^- \times [\text{NO}_3^-])) / [\text{DON}] \quad (3\text{-}1)$$

Using mineral N obtained by *in situ* exchange resins should be superior to salt extractions because the exchange resins are time integrated. Inorganic and amino acid N pools have a very short mean residence time associated with episodic production and removal (Jones & Kielland 2002; Booth *et al.* 2005). These 'events' and their associated isotope discrimination patterns may be missed by single time-point soil extractions that may vary from those from exchange resins (Koba *et al.* 2003) and which may change markedly within days to months (Evans 2007). For instance, organic N extracted with ion exchange resins reduces the potentially severe isotope fractionation (ε16.5‰) of NO_3^- associated with typical salt extractions (Hales & Ross 2008). Furthermore, fractionation due to NH_4^+ diffusion gradients around resins was unlikely because Biorad® AG 50W-X8 exchange resins have been shown to produce negligible effects on isotope fractions even in the presence of potentially interfering cations and dissolved organics (Lehmann *et al.* 2001).

Phospholipid fatty acid analyses (PLFA) were conducted on original frozen soil subsamples. From one to three 1-5 g wet weight subsamples were analyzed for PLFA's from the composited soil core replicates used to extract N. Nine of the 31 plots were run in triplicate. This process involved an initial lipid extraction, fractionation, and successive elution (Frostegård *et al.* 1991) followed by conversion of the methanol

fraction into free methyl esters by mild alkaline methanolysis, and then analysis on a gas chromatograph with a flame ionization detector and a 50 m HP5 capillary column (Frostegård *et al.* 1993). The PLFA 18:2ω6,9 was regarded as an indicator of total fungal biomass (Frostegård & Bååth 1996) and is likely to be comprised mainly of ECM fungi in spruce forest as the majority of the microbial biomass in boreal forest ecosystems is known to be predominantly ECM fungi (Allison *et al.* 2007; Lindahl *et al.* 2007; Taylor *et al.* 2010). We used the first PCA axis (contained 57% of the variability) containing both fungal and bacterial PLFA's because of the capacity to incorporate additional microbial changes. Regardless, the first PCA axis was positively correlated with fungal:bacterial PLFA ratios (R^2 = 0.83) and total mol% of the fungal PLFA 18:2w6,9 (R^2 = 0.93) indicating that the variables were interchangeable. Of the thirty PLFA's retained for a PCA, the PLFA's i15:0, a15:0, 15:0, 10Me16:0, i17:0, a 7:0, cy17:0, 10Me17:0, br18:0, 10Me18:0, and cy19:0 were used as an indicator of bacterial biomass and the PLFA 18:2ω6,9 as an indicator of total fungal biomass (Frostegård & Bååth 1996). Changes in the PLFA 18:1ω9 was also used as corroborated evidence for changes in fungal biomass, although it was not used in calculation of a fungal:bacterial biomass index.

Statistical Analyses

Linear regressions were constructed on the following response variables: foliar δ^{15}N, %N, and %P; soil δ^{15}N values of DON and NH_4^+; standing aboveground biomass of black spruce; and belowground biomass of fungi. For each model we wished to reduce our explanatory variables to prevent inflation of Type I error rates (Harrell 2001). In particular, most soil fertility variables were reduced to single PC axes. This

partial variable reduction allowed specific variables of interest to be retained in subsequent models depending on the response variable being modeled. For instance, when modeling foliar N content, DON and resin-extractable mineral N were retained as independent predictors whereas the remaining soil fertility variables were reduced to a single PC axis. Similarly, for foliar P, resin extractable PO_4^- was withheld from it's PCA, and in the case of foliar $\delta^{15}N$, the $\delta^{15}N$ values of the soil N forms were withheld. Fungal biomass was withheld from all soil fertility PC analyses so that the explanatory power of this variable could be independently assessed. Predictor variables were standardized by subtracting from the mean and dividing by the standard deviation (Schielzeth 2010). Independence of explanatory variables was assessed with scatter plot matrices and variance inflation factors to assure a lack of multicolinearity and to detect severe outliers. PCA was conducted using JMP® 8.0.2 (SAS Institute Inc., Cary, NC, USA).

Second-order bias-corrected Akaike information criterion ($\Delta_i = AICc_i - AICc_{min}$) was used to rank models along, with model probabilities ($\omega_i = \exp[-½\Delta_i] / \sum\exp[-½\Delta]$), because it is generally regarded as an unbiased estimator that assess relative model fit, penalizes over-parameterization, and allows multiple working hypotheses to be simultaneously evaluated (Burnham & Anderson 2004; Andersen 2008). In contrast, fitting all possible models or using stepwise model selection has potential to inflate Type I error rates and to fail selection of the most informative model (Whittingham *et al.* 2006; Andersen 2008) but see Murtaugh (2009). Graphical diagnostics of residuals against fitted values and sample quantile against theoretical quantile plots were used to assess underlying distributional assumptions for all high-ranking models. Model fitting and

diagnostics were performed using the R statistical environment (2.9.2, The R® Foundation for statistical computing 2009).

Isotope Mass Balance

Building upon the $\delta^{15}N$ mass balance models described previously for arctic tundra (Hobbie & Hobbie 2006; Hobbie & Hobbie 2008; Yano *et al.* 2010), we estimated the proportional dependence of black spruce for ECM-derived N iteratively within each of our sites using the following mathematically under-determined equations:

$$\delta^{15}N_{available} = f_{NH4} \bullet \delta^{15}N_{NH4} + f_{DON} \bullet \delta^{15}N_{DON} \quad (3\text{-}2)$$

$$\delta^{15}N_{available} = (1 - T_r) \bullet \delta^{15}N_{fungi} + T_r \bullet \delta^{15}N_{transfer} \quad (3\text{-}3)$$

$$\delta^{15}N_{plant} = \delta^{15}N_{available} - \Delta_f \bullet (1 - T_r) \bullet f \quad (3\text{-}4)$$

$$\delta^{15}N_{fungi} = \delta^{15}N_{available} + \Delta_f \bullet T_r \quad (3\text{-}5)$$

where f_{NH4} and f_{DON} in equation (3-2) refer to the fraction of these soil N forms contributing to the available N pool, T_r in equation (3-3) refers to the proportion of total fungal N that is transferred to host plants, $\delta^{15}N_{transfer}$ refers to the $\delta^{15}N$ value of the transfer compounds produced by ECM fungi, and f in equation (3-4) refers to the proportion of plant N supplied by fungi and Δ_f refers to the fractionation magnitude associated with transamination of soil N within ECM fungi (Hobbie & Hobbie 2008).

We quantitatively constrained the mass balance solutions in the following way: (a) The fraction of plant N delivered by ECM fungi (f) cannot exceed 100% of the trees' N supply; (b) Mixtures of the different N forms occur at 10% increments; (c) Δ_f was defined as 8-10‰ based on laboratory, field, and meta-analyses as described in detail elsewhere (Hobbie & Hobbie 2006; Hobbie & Hobbie 2008); and, (d) similar to Yano *et*

al. (2010) the proportional use of NH_4^+ (f_{NH4}) was initially assumed to contribute less or equally weighted contribution (e.g. 10-50%) of total N uptake by plants or fungi, however higher proportions were permitted in order to meet constraint (a) where necessary. High DON reliance by black spruce is justified because mineral N supplies in boreal ecosystems cannot typically meet annual tree N requirements (Ruess *et al.* 1996; Read *et al.* 2004; Valentine *et al.* 2006). The $\delta^{15}N$ values from root tissue were used instead of those from foliage because this permits accounting of internal plant fractionation (Robinson 2001). In addition, IsoError® 1.04 (Phillips & Gregg 2001) was used to estimate proportional mixing and to generate confidence intervals associated with a two source pool (e.g. ECM fungal derived and direct uptake of N) mixture using data averaged across all plots.

Results

Tree Biomass and Soil Fertility in Black Spruce Stands

Black spruce exhibited a broad range of isotopic, elemental, and biomass values across the plots (Table 3-1). For instance, above-ground black spruce biomass ranged from 2.8 to 100 Mg ha^{-1} in part due to changes in soil fertility. Soil fertility variables such as active layer depth, soil moisture, pH, CEC, C:N, and mineral and organic nutrient contents (Table 3-2) were reduced to a single PC axis that accounted for substantial variation in black spruce biomass (R^2_{adj} = 0.33, P = 0.003). Fungal biomass, in contrast, was not retained in high-ranking explanatory models (Table 3-3). Upon further inspection, much of the explanatory power of the soil fertility PC axis can be attributed to soil C:N values alone (R^2_{adj} = 0.20, P = 0.008), not active layer depth, pH, or soil moisture, as might be expected under growth-limiting conditions.

The pool of soil dissolved organic N (DON) ranged from 148 to 602 $\mu g\ g^{-1}$ in organic soils, a value six times greater than in mineral soils (Table 3-2). Resin exchangeable NH_4^+ and NO_3^- concentrations were extremely low in many plots, ranging from 0.00 to 3.70 and 0.00 to 0.55 ng N g^{-1} resin d^{-1}, respectively (Table 3-2). Resin exchangeable NO_3^- levels in particular were undetectable in all but seven plots, and in those seven, compositing of replicates was necessary to provide sufficient N for ^{15}N measurements using the denitrifier method. NH_4^+, in contrast, were at detectable concentrations in all but three of the plots. Resin exchangeable PO_4^- concentration was nearly 12 times higher and 16 times more variable than NH_4^+ (Table 3-2). The greater variability could be due to variation in depth of the relatively unweathered parent material and low PO_4^- mobility in organic soils.

Black Spruce Foliar $\delta^{15}N$, %N, and %P Patterns Across the Landscape

Foliar $\delta^{15}N$ values in black spruce trees were strongly correlated with both foliar N (R^2 = 0.46, P < 0.001) and foliar P content (R^2 = 0.51, P < 0.001) across the 31 plots (Figure 3-1). Collectively, foliar $\delta^{15}N$ values in this single species varied by 6.5‰, a range that encompasses the lower third of foliar $\delta^{15}N$ values observed from 9,757 plant samples world-wide (Craine *et al.* 2009). Root $\delta^{15}N$ values were on average 2.35‰ less depleted than tree needles (Figure 3-2) and were positively correlated with foliar $\delta^{15}N$ values although not strongly so (R^2 = 0.30, P = 0.002). The slope of the relationship was not 1:1 as expected either ($\delta^{15}N_{foliar} = -4.69 + 0.51\delta^{15}N_{root}$). Instead root and foliar $\delta^{15}N$ values converged at the least negative plant $\delta^{15}N$ values (Figure 3-2).

Multiple regression model selection indicated that foliar $\delta^{15}N$ values were best explained by the 1st soil fertility PC axis (R^2_{adj} = 0.21), and in two of the three highest

ranking models, $\delta^{15}N_{NH4}$ alone or with $\delta^{15}N_{DON}$ were also retained (R^2_{adj} = 0.25 and 0.30, respectively; Table 3-3). These models did not include foliar N content (which doubles the R^2_{adj}) because soil variables were of primary mechanistic interest. Foliar N content, in turn, was best explained by models containing the DON content of organic soils (μg g^{-1} soil), either singly (R^2_{adj} = 0.25) or in combination with either PLFA based fungal biomass (R^2_{adj} = 0.35), resin extracted mineral N (ng g^{-1} resin, R^2_{adj} = 0.25), or a soil fertility PC axis excluding soil N pools (R^2_{adj} = 0.31; Table 2-3). The model with the best explanatory power and weight included both the DON and fungal biomass, expressed as the first PC of the PLFA pattern (R^2_{adj} = 0.35, ω_i = 0.21). Variation in black spruce foliar P content (g g^{-1}) was best explained by models containing both soil PO_4^+ (ng g^{-1} of resin day^{-1}) and either a soil fertility PC axis or an interaction with hyphal ingrowth biomass (R^2_{adj} = 0.59-0.60; Table 3-3). The most parsimonious candidate model of lesser model probability (ω_i = 0.19 vs. 0.30) contained only the soil fertility principle component without losing substantial explanatory power (R^2_{adj} = 0.56).

Sporocarp $\delta^{15}N$ and Fungal Biomass

Using a discriminant model based on over 800 sporocarp $\delta^{15}N$ and $\delta^{13}C$ global patterns (Mayor *et al.* 2009) we confirmed that all sporocarps used in our analysis were likely to be ectomycorrhizal at >90% probability (data not shown). The majority of analyzed sporocarps were species of *Cortinarius, Dermocybe, or Russula,* based on macroscopic characters. On average the ECM sporocarps were 13.8‰ more ^{15}N-enriched than black spruce foliage (Table 3-1), and, on average, similar to the mean for ECM sporocarps globally (Mayor *et al.* 2009) and elsewhere in Alaska (Clemmensen *et al.* 2008; Hobbie *et al.* 2009). Sporocarp $\delta^{15}N$ values were negatively correlated with g

DON m^{-2} in organic soil (R^2 = 0.39; Figure 3-3), but not mineral N (as measured from ion exchange resins). Diagnostics of the two influential values were not indicative of gross outliers (Cook's distance <0.2, overall Leverage <0.5), although removal of the two values with the highest g DON m^{-2} reduces the R^2 to 0.17. Sporocarp $\delta^{15}N$ values were not explicitly modeled in a multivariate context because of low sample size (N = 17) limiting the number of predictor variables available to just two, although scatterplot matrices indicated no correlation with soil N $\delta^{15}N$ values in a univariate context.

The estimates of fungal biomass in organic soils, represented as a PLFA-based principle component, were positively correlated with C:N ratios (g g^{-1}) of the organic soil (R^2 = 0.66, Figure 3-4) and were uncorrelated with hyphal ingrowth or other soil fertility metrics. Subsequent model selection confirmed that fungal biomass increased across the landscape in relation to soil C:N ratio alone (ω_i = 0.57). More complex models containing DON (μg g^{-1} soil) and CEC (meq) were less probable (ω_i = 0.19 or 0.18, respectively; Table 3-3). Of the four hyphal ingrowth bags with appreciable colonization, mean $\delta^{15}N$ values indicated ~6‰ depletion (mean = 0.35‰) and on average 1.7% less N than ECM sporocarps.

$\delta^{15}N$ Patterns of Soil N Forms Across the Landscape

As seen graphically, 26 of the 31 plots had more enriched mean $\delta^{15}N_{DON}$ values than $\delta^{15}N_{NH4}$ yet no covariance was found and the few $\delta^{15}N_{NO3}$ values that could be obtained lacked any pattern (Figure 3-5). Post hoc paired t-tests indicated that average $\delta^{15}N_{DON}$ and $\delta^{15}N_{NH4}$ values differed from each other and that of the $\delta^{15}N_{NO3}$ and $\delta^{15}N$ of bulk organic soil (P < 0.001). Overall, the ranking pattern observed was: $\delta^{15}N_{DON}$ > $\delta^{15}N_{NH4}$ > $\delta^{15}N_{NO3}$ ≈ $\delta^{15}N_{soil}$ (Figure 3-5). Despite overall differences in many soil N-$\delta^{15}N$

values, the variation across sites was unrelated to the parameter combinations used in the regression analysis and was uncorrelated with plant or fungal $\delta^{15}N$ values (Figure 3-2 & 3-5). The predictor variables used were based on what were likely to be direct inputs to either the DON or NH_4^+ pool measured in our system: the biomass of fungi, black spruce roots, and foliage for $\delta^{15}N_{DON}$; and $\delta^{15}N_{DON}$ for $\delta^{15}N_{NH4}$.

Because enrichment factors ($\varepsilon = \delta^{15}N_{foliar} - \delta^{15}N_{soil}$) have been employed to standardize comparisons of foliar $\delta^{15}N$ across sites (Garten Jr. & Van Miegroet 1994; Martinelli *et al.* 1999; Vervaet *et al.* 2002; Amundson *et al.* 2003; Kahmen *et al.* 2008; Takebayashi *et al.* 2010) we evaluated the following permutations: $\varepsilon = \delta^{15}N_{foliar} - \delta^{15}N_{soil}$, $\delta^{15}N_{DON}$, and $\delta^{15}N_{NH4}$. Correlation among these ε factors and metrics of soil N availability (e.g. DON, resin NH_4^+, and C:N) were then examined in an attempt to differentiate N-rich from N-poor sites. Only the ε based on $\delta^{15}N_{foliar} - \delta^{15}N_{NH4}$ exhibited a positive relationship with resin $[NH_4^+]$ ($R^2_{adj} = 0.16$, $P = 0.014$; $[NH4_{res}] = 2.42 + 0.133\varepsilon_{15Nfoliar\text{-}15NH4}$).

Modeling Fractionation of ^{15}N in ECM Fungi and Transfer to Black Spruce

Using measured $\delta^{15}N$ values of black spruce roots, soil N moieties, and ECM sporocarps (where available) from each plot left only two factors to define in equations 3-2 to 3-5. First, we iteratively set the fractionation effects attributed to ECM fungal transamination of transfer compounds to Δ = 8–10‰ (Hobbie & Hobbie 2006; Hobbie & Hobbie 2008) and the proportional use of NH_4^+ (f_{NH4}) was *a priori* assumed minor in accordance with previous modeling efforts in Alaskan tundra (Yano *et al.* 2010) and for reasons related to the DON abundance and use in black spruce ecosystems previously discussed. However, f_{NH4} was increased to achieve sensible values in many plots (e.g.

if proportional supply of plant N from ECM fungi was greater than 100%). Owing to the extremely low availability of NO_3^- and its detection in only seven plots, it was omitted from all but four subsequent mass balance models where solutions required its inclusion and values were available (Appendix C-3). Where sporocarp $\delta^{15}N$ values were unavailable we first attempted to use the average value of all the plots (6.5‰) as there was no trend from which to interpolate (Figure 3-2). However, in many instances this value was too low to achieve less than 100% proportional supply of plant N from ECM fungi. This constraint required assignment of ECM sporocarp $\delta^{15}N$ values equivalent to the most enriched sporocarps collected from one of our plots (11‰) in 15 of the mass balance models (Appendix C-3). Plant, ECM sporocarp, and soil DON, NH_4^+, and NO_3^- $\delta^{15}N$ value end members in mixing equations were measured in replicate from each plot but variability among replicate measurements of N sources was not propagated through the models because of mathematical uncertainty associated with three sources contributing to one mixture. If measurement variability was included then the range of potential solutions would likely increase, yet the average ECM derived N would decrease slightly in order to prevent upper confidence interval bounds from exceeding 100%.

Plot specific solutions suggest that the fraction of tree N nutrition derived from ECM fungi (f) ranged 0.6–1.0 (average = 0.9) and the fraction of fungal N that was transferred to host plants (T_r) ranged 0.08-0.65 depending on the proportion of NH_4^+ contributing to total N supply (Appendix C-3). These plot specific f values were uncorrelated with our metrics of soil fertility (DON, NH_4^+, pH, moisture, CEC, active layer depth) or metrics of tree or fungal biomass. However, f values were positively

correlated with $\delta^{15}N_{NH4}$ (R^2_{adj} = 0.16, P = 0.01, y = 0.85 + 0.017χ) and negatively correlated with both sporocarp and root $\delta^{15}N$ values (R^2_{adj} = 0.39, P = 0.004, y = 1.07 - 0.025χ; R^2_{adj} = 0.16, P = 0.02, y = 0.74 - 0.03χ, respectively).

Next we sought to conduct a single mass balance model using IsoError®. End member $\delta^{15}N$ values of the mixture were simply the $\delta^{15}N_{root}$ value from plot averages, whereas the two sources represented the $\delta^{15}N$ value of soil available N derived from ECM fungi ($\delta^{15}N_{available} - \Delta 9‰$) where $\delta^{15}N_{available}$ was estimated from equation (3-2) with a priori N contributions conservatively set to 70% NH_4^+ and 30% DON. The contribution of NH_4^+ to total plant N nutrition could not be below 70% without exceeding estimates of plant N dependency on ECM fungi being greater than 100% of total plant N nutrition. In addition, fractionation magnitudes associated with the formation of N transfer compounds by ECM fungi had to be ≥9‰. With these parameters, the average (± SE) fraction of total plant N nutrition derived from ECM fungi was 98.9% ±0.06 (C.I. 86-100%) with the remainder derived from direct plant root uptake.

Discussion

Black Spruce Elemental Patterns

The 6.5‰ variation in foliar $\delta^{15}N$ values in this study encompassed the lower third of foliar $\delta^{15}N$ values observed from 9757 plants (900 sites, 1103 taxa) collected world-wide (Craine *et al.* 2009). Comparable magnitudes of variation in foliar $\delta^{15}N$ values were recorded in an additional 40 plots from central Alaska (M.C. Mack, unpublished data) and along a latitudinal transect spanning 2º (~150 km) from the Yukon River to the north slope of Alaska (Hobbie *et al.* 2009). These studies suggest that the black spruce

$\delta^{15}N$ patterns observed in this study are representative of a much larger geographical area.

In agreement with theoretical expectations, linear model results supported the use of both foliar $\delta^{15}N$ and %N as indices of site fertility. The best explanatory variable(s) for foliar N content was DON (g^{-1}) content in the organic layer, either alone or in combination with fungal biomass or mineral N. This finding provides further support that DON is likely an important N source for these boreal forest trees (Näsholm *et al.* 2009). Foliar $\delta^{15}N$, in turn, was best explained by the first soil fertility PC axis, either with or without specific $\delta^{15}N$ values of soil N moieties (Table 3-3). In both instances parsimony warrants the use of models with single predictor variables, but inclusion of fungal biomass increased the explanatory power of foliar N content from R^2_{adj} = 0.25 to 0.35 supporting the idea that ECM fungi are important for black spruce N nutrition and predicting additional plot variability may warrant the additional effort involved with the inclusion of PLFA data.

Strong N-limiting growth conditions of black spruce trees were indicated by an average needle N:P ratio of 7 ($g\ g^{-1}$), as found in plants growing from other high latitude terrestrial ecosystems (Güsewell 2004). It is therefore likely that the close correlation between $\delta^{15}N$, P, and N concentrations in needles (Figure 3-1) represents a stoichiometric relationship where plant P trails behind foliar N concentration. Although P is seldom considered to limit plant growth other than in water-saturated boreal and tundra ecosystems, there appear to be non-flooded conditions under which soil microbes experience P limitation (Giesler *et al.* 2004) which, in turn, may influence N acquisition by black spruce if extracellular enzyme production by ECM fungi is impaired

(Alvarez *et al.* 2009). Foliar %P model selection indicated that the resin exchangeable PO_4^+ was informative in two of the three highest ranking models and that complex models including a soil fertility PCA axis or an interaction with hyphal ingrowth biomass were also informative (Table 3-3). This finding may be due to the well documented advantage fungal hyphae imbue on plant P supply owing to enhanced surface area for nutrient absorption (Smith & Read 2008).

We observed black spruce needles becoming less ^{15}N depleted with greater N content (Figure 3-1). This pattern is explained by the fungal fractionation hypothesis (Hobbie *et al.* 2009) where reduced dependency of black spruce on ECM-derived N at relatively higher levels of N availability would reduce ^{15}N depleted N transfer compounds from influencing foliage. This process of varying dependency on ECM-derived N from fungi is supported by laboratory (Hobbie *et al.* 2001b; Hobbie & Colpaert 2003), greenhouse (Hobbie *et al.* 2001a), and field observations along successional chronosequences (Hobbie *et al.* 2005; Compton *et al.* 2007; Hobbie & Hobbie 2008). The same correlative pattern has also been found in other high latitude ECM forming tree species such as *Abies lasiocarpa* and *Picea glauca* in British Colombia (Kranabetter & MacKenzie 2010), *Picea glauca* in northern Alaska (Schulze *et al.* 1994), *Pinus strobus* in Rhode Island (Compton *et al.* 2007), and several other taxa in lower Alaska including species of *Picea*, *Populus*, *Salix*, and *Tsuga* (Hobbie *et al.* 2000). However, similar correlative patterns have also been described from trees associating with arbuscular mycorrhizal fungi along gradients of succession (Davidson *et al.* 2007), precipitation (Schuur & Matson 2001), and soil age (Vitousek *et al.* 1989) among others (Pardo *et al.* 2006; Craine *et al.* 2009). The presence of these patterns in non-ECM

associating trees suggests additional mechanisms may be at work. Therefore, it seems prudent to first account for the type of root symbionts prior to assessment of additional underlying mechanisms of foliar $\delta^{15}N$ patterns, such as soil N status or climate (Högberg 1997; Craine *et al.* 2009; Pardo & Nadelhoffer 2010).

Internal fractionation, such as during N translocation from root to needle, were indicated in black spruce by large differences in root and foliar $\delta^{15}N$ values (Figure 3-2). Internal fractionation in plants can occur during resorption of foliar N, incorporation into organic compounds, or during translocation of N from organ to organ, and it is important to consider these effects because they could obscure the ^{15}N signature of the N sources (Evans 2001; Robinson 2001). Complicating interpretation, however, was the finding that observed differences were not consistent across plots; they ranged from 0 to 4‰ and converged where tissue $\delta^{15}N$ values were the least ^{15}N depleted (Figure 3-2) and N content was highest (Figure 3-1). In general, the average magnitude of the isotopic fractionation (Δ2.35‰) was in line with expectations based on patterns observed for other plants growing in mesic conditions (Gebauer 1993; Näsholm 1994; Yoneyama 1995; Högberg 1997; Evans 2001) although exceptions do exist for unknown reasons. For instance, in Austrian spruce (*Picea abies*) plantations, no $\delta^{15}N$ differences between root and needle were found but a 1.5‰ difference was found in beech (Pörtl *et al.* 2007). These patterns illustrate that internal plant fractionating processes are not necessarily consistent within or across species and may diminish under conditions relative ^{15}N enrichment or enhanced N availability. For this reason assignment of a single internal fractionation magnitude in isotope modeling efforts may not be accurate under all conditions.

What caused the observed reduction in (or loss of) internal ^{15}N fractionation in black spruce? In other tree species internal ^{15}N fractionation shifts in response to water stress and topographic position (Bai *et al.* 2009), or under conditions where N supply exceeds N demand, particularly when NO_3^- is the main N source (Evans 2001). However, we found no correlation among soil moisture or soil N availability with either foliar or root $\delta^{15}N$ values and NO_3^- levels were exceedingly low in our stands, often below detection limits (Table 3-2). Furthermore, in both black spruce and temperate oaks there is no evidence for ^{15}N discrimination during resorption of foliar N prior to abscission (Kielland *et al.* 1998; Kolb & Evans 2002). Eliminating these causes leaves varying N processing and/or internal allocation patterns as sources of observed ^{15}N fractionation in black spruce. Most processes fractionate against ^{15}N, suggesting increased metabolic processing in the roots could cause the greater ^{15}N depletion observed in black spruce in the most ^{15}N depleted plots (Dijkstra *et al.* 2008). These processes could include, but are not limited to: greater incorporation of NH_4^+ into glutamine via glutamine synthetase (Werner & Schmidt 2002); greater levels of transamination in roots relative to foliage during growth (Shearer & Kohl 1986; Evans 2001); and/or, altered amounts of organic N transported through the xylem or leaked from roots (Dijkstra *et al.* 2003; Yoneyama *et al.* 2003). Without compound-specific phloem and xylem ^{15}N analyses, however, we cannot determine which processes are driving the observed patterns of shifting internal ^{15}N fractionation in black spruce.

Fungal Biomass and Sporocarp $\delta^{15}N$ values

We observed a tendency toward ^{15}N enrichment of ECM sporocarps under conditions of relatively low soil DON content (R^2 = 0.39), a pattern driven in part by two

high DON measurements (Figure 3-3). Assuming black spruce N demand increases in response to low DON content, ^{15}N enrichment of ECM sporocarps could result from greater retention of ^{15}N enriched N during the increased production and transfer of ^{15}N depleted N to black spruce (Högberg *et al.* 1999a; Hobbie & Colpaert 2003). This is a corollary to the hypothesis elicited above to explain the observed pattern of foliar and root $\delta^{15}N$. Retention of soil DON content and fungal biomass in models explaining foliar N further support the role of soil DON content in black spruce dependency for ECM derived N (Table 3-3). However, there was no negative correlation among sporocarp and foliar $\delta^{15}N$ values (Figure 3-2) as would be expected to corroborate this explanation. We urge caution in accepting these patterns due to the small replication of opportunistically collected sporocarps from plots. The $\delta^{15}N$ values are based on sporocarp samples that were replicated in only three of 17 sites. Single sporocarp $\delta^{15}N$ values may be inaccurate representations of the $\delta^{15}N$ values of the total fungal community if the sampled species accesses either a unique soil substrate or horizons (Dickie *et al.* 2002; Tedersoo *et al.* 2003; Lindahl *et al.* 2007; Wallander *et al.* 2009), or is of an unrepresentative age or exploration type (Trudell *et al.* 2004; Hobbie & Agerer 2009). It has also been suggested that organic N use itself, independent of the interaction with host trees, can contribute to ^{15}N enrichment of fungi relative to those constrained to only mineral N sources (Henn & Chapela 2004; Trudell *et al.* 2004). This mechanism, however, has yet to be demonstrated in the field and likely wouldn't account for variation across the gradient if the majority of ECM taxa associated with black spruce access DON.

Variation in fungal biomass was well explained and positively correlated with the parsimonious regression model containing only the C:N ratio of organic soil (R^2 = 0.65; Table 3-3); an index that varied 20 units (25 to 45) largely in response to changes in soil N content. Positive relationships between fungal biomass and C:N ratios in surface soils have also been described from other forests as well (Nilsson *et al.* 2005; Smith & Read 2008; Wallander *et al.* 2009).

Of the 180 hyphal ingrowth bags deployed throughout the growing season, very few produced enough tissue for δ^{15}N measurement. This result could be due to the lack of growth-limiting mineral nutrients contained within the acid washed sand and the relatively short incubation period (Wallander *et al.* 2004; Hendricks *et al.* 2006). In accordance with our observations, hyphae in other ecosystems dominated by ECM plants have also been observed to be ^{15}N depleted relative to co-occurring ECM sporocarps, possibly due to N being preferentially reallocated from 'evacuated' hyphae to protein and N-rich sporocarps during development (Wallander *et al.* 2004; Clemmensen *et al.* 2006; Boström *et al.* 2007). This pattern was recently explained using two pool mass balance equations derived to account for δ^{15}N patterns of differing ECM fungi exploration types (Hobbie & Agerer 2009). It is also possible that the small amounts of harvestable mycelium contained in our ingrowth bags could have been from fast growing saprotrophic fungi know to have δ^{15}N values that are less enriched than ECM fungi (Mayor *et al.* 2009).

Fungal biomass estimates range from 2.0×10^3 kg ha^{-1} in tussock tundra to 9.5 kg ha^{-1} temperate forest (Clemmensen *et al.* 2006), and 4.8×10^3 kg ha^{-1} in spruce to 5.8×10^3 kg ha^{-1} in mixed boreal forests (Wallander *et al.* 2004). These large pools of

ECM biomass are thought to contribute to observed ^{15}N-enrichment of soil N with depth, forest maturation, and labile DON pools (Hobbie & Ouimette 2009; Wallander *et al.* 2009; Takebayashi *et al.* 2010). Microbial processing of these fungi derived N pools may itself lead to further ^{15}N-enrichment as well (Kramer *et al.* 2003; Dijkstra *et al.* 2008). Foliar leaching of N and P is largely intercepted by feather and sphagnum mosses (Weber & Van Cleve 1984; Chapin III *et al.* 1987) leaving soil organic matter, and the resulting labile N, to be derived from slowly decomposing black spruce and moss litter (Smith *et al.* 1999) along with large quantities of ^{15}N enriched fungal biomass in spruce forests (Högberg & Högberg 2002).

δ^{15}N Patterns of Soil N Moieties Across the Landscape

Measuring specific forms of N in replicate across the landscape revealed substantial variability but different mean δ^{15}N values of soil N moieties (Table 3-2, Figure 3-5). The extractable DON pool was more ^{15}N enriched than resin extractable NH_4^+ and bulk soil organic matter in 77% and 100% of the plots, respectively. Despite these overall differences, variability among δ^{15}N values of N forms were not explained with the parameters used in regression models (Table 3-2) suggesting soil N isotope cycling may vary independently of plant N cycles. This is in agreement with models suggesting only inputs, hydrological leaching, and gaseous losses influence soil δ^{15}N values (Amundson *et al.* 2003; Bai *et al.* 2009). As *Pleurozium* and *Hylocomium* mosses in eight of our plots had relatively consistent mean (±SE) δ^{15}N values of -2.98 ±0.31‰, (unpublished data), inputs were likely from belowground sources. The δ^{15}N values of individual soil N moieties were also uncorrelated with those from plant tissues or sporocarps (Figure 3-2) indicating that source N cannot simply be traced without

accounting for fractionation along the way. These complex and uncorrelated $\delta^{15}N$ patterns among the pools of cycling N support the claim that $\delta^{15}N$ values of soil N, and especially bulk soil organic matter, may not approximate plant $\delta^{15}N$ values in many N-limited ecosystems (Evans 2001; Pardo *et al.* 2006).

As mentioned, many researchers have used ε factors to link observed plant and bulk soil $\delta^{15}N$ values with the N status (Emmett *et al.* 1998), net nitrification (Garten Jr. & Van Miegroet 1994), or mineralization potential (Kahmen *et al.* 2008). In black spruce forest, only ε based on the difference between $\delta^{15}N_{foliar}$ and $\delta^{15}N_{NH4}$ exhibited a positive relationship with resin NH_4^+ availability (R^2 = 0.19, P = 0.01). However, the low total variation explained and lack of correlation among ε based on bulk soil organic matter $\delta^{15}N$ does not support continued use of enrichment factors to adjust foliar $\delta^{15}N$ values across sites prior to elucidation of underlying soil N isotope patterns. We suggest that use of these corrections may mask natural complexity and limit accurate modeling of ultimate causes of plant $\delta^{15}N$ variability in many ecosystems, particularly those dominated by ECM-forming trees.

Black spruce soil N $\delta^{15}N$ measurements (Table 3-2) correspond with previously described patterns from tropical, temperate, and tundra ecosystems as follows: $\delta^{15}N_{DON} > \delta^{15}N_{amino\ acid} > \delta^{15}N_{NH4} > \delta^{15}N_{bulk} > \delta^{15}N_{NO3}$ (Houlton *et al.* 2007; Takebayashi *et al.* 2010; Yano *et al.* 2010). The $\delta^{15}N_{DON}$ may be enriched relative to other N forms due to inputs of ^{15}N enriched materials, such as fungal biomass discussed above, or preferential use and export of isotopically light N compounds (Schmidt *et al.* 2006). However, this latter effect would require relatively ^{15}N depleted decomposition products to be removed from the system through leaching, volatilization, or stabilization in

underlying permafrost as previously hypothesized (Hobbie *et al.* 2009). The $\delta^{15}N$ value of NH_4^+ is generally considered isotopically lighter than its substrate due to enzymatic preference for ^{14}N-bound polymeric N (Silfer *et al.* 1992; Nadelhoffer & Fry 1994). This pattern was supported in 77% of our 31 plots yet the magnitude of the differences varied appreciably (Figure 3-5). In addition to variability attributed to the source DON pool, NH_4^+ is also being incorporated into organic N mainly via the glutamine synthetase pathway, which may be fractionating against ^{15}N (Werner & Schmidt 2002). In effect, increased demand for NH_4^+ by soil microbes could increase the $\delta^{15}N$ values of residual NH_4^+ and as it cycles through microbial populations it may be subjected to multiple competing reactions, each with different fractionation factors (Evans 2007; Hobbie & Ouimette 2009) that could contribute to the complex soil patterns in black spruce forest.

Other studies report amino acid $\delta^{15}N$ values ranging from -8.7 to +8.1‰ (Melillo *et al.* 1989; Silfer *et al.* 1992; Ostle *et al.* 1999; Werner & Schmidt 2002; Bol *et al.* 2008; Yano *et al.* 2010). Furthermore, as soil organic matter increases in recalcitrance (e.g. aliphaticity), it has been observed to become gradually ^{15}N enriched – ranging from -6 at low aliphaticity to 13‰ at high aliphaticity (Kramer *et al.* 2003). This potentially broad range of DON substrate $\delta^{15}N$ values suggests compound-specific absorption could substantially influence black spruce $\delta^{15}N$. In tussock tundra of northern Alaska amino acid $\delta^{15}N$ values were ^{15}N depleted relative to the total DON pool by as much as 10‰ (Yano *et al.* 2010). However, many species of ECM forming fungi can enzymatically degrade large molecular weight DON compounds such as chitin, proteins, and peptides (Abuzinadah & Read 1986; Abuzinadah & Read 1988; Lindahl & Taylor 2004; Read *et al.* 2004; Nygren *et al.* 2007; Bödeker *et al.* 2009; Talbot & Treseder 2010) that are

typically more ^{15}N enriched than amino acids (Werner & Schmidt 2002). Furthermore, black spruce forest soils have some of the highest protease activities of all taiga ecosystems (Kielland *et al.* 2007) and despite the high volume of protein binding tannins present in boreal soils (Joanisse *et al.* 2009), it is likely that access to numerous DON compounds would smooth out substrate-based isotopic differences. Therefore, regardless of the small proportion of directly absorbable amino acid N in the DON pool (Chalot *et al.* 2002; Jones & Kielland 2002; Jones *et al.* 2005a), the $\delta^{15}N_{DON}$ in black spruce forest is likely to match the combined cumulative $\delta^{15}N$ value of the numerous organic N compounds accessed by black spruce and their associated ECM.

Modeling N Transfer to Black Spruce

Both mass balance methods, one based on detailed mechanistic modeling (Hobbie & Hobbie 2006) applied to plot-by-plot estimates, the other based on simple yet sensible a priori two-pool mixtures and fungal fractionation effects, illustrate a high dependency of black spruce on ECM derived N (f = 60–100%, averaging 89% or 98.9%, respectively; Appendix C-3). Furthermore both modeling efforts illustrate the large contribution DON (30-49% on average) must make to meet N requirements for growth in these strongly N limited boreal forest ecosystems. The resulting dependencies on ECM derived N (f) from individual modeling efforts indicated a positive correlation with the $\delta^{15}N_{NH4}$ source and negative correlations with sporocarp and root $\delta^{15}N$ values. The strongest negative correlation with sporocarp $\delta^{15}N$ values appears at first glance to run counter to the fungal fractionation hypothesis where more enriched sporocarps might indicate greater proportional delivery of N and retention of ^{15}N enriched N. However, the particularly enriched sporocarp $\delta^{15}N$ values may also

merely reflect specific peculiarities associated with those specific fungal taxa such as: access to enriched substrates, growth in deeper soil horizons, or, as previously mentioned, they could be generally unrepresentative of the greater ECM fungal community in those plots.

If the enriched DON $\delta^{15}N$ values we measured were not representative of the proportion of DON that is actually bioavailable, and that smaller proportion is isotopically depleted relative to the total DON pool, then our estimates of ECM fungal delivery of N (f) are exaggerated. This possibility exists because the actual $\delta^{15}N_{available}$ end member would be closer to that of the black spruce root $\delta^{15}N$ value requiring less N transfer through ECM fungi to account for isotopic differences between source and sink. Recent partitioning of soil DON pool $\delta^{15}N$ values (Yano *et al.* 2010), while a step in the right direction, does not directly address this issue because the question of what constitutes the bioavailable portion of DON is not easily determined in these ECM dominated organic soils.

Conclusions

The mixing model and multiple regression data support the idea that ECM fungi are integral to meeting the N requirements of black spruce and DON contributes directly to black spruce N nutrition without complete mineralization prior to uptake. These findings are sensible given the strong selective pressure for access to this abundant N pool under such severe N-limiting conditions (Neff *et al.* 2003; Schimel & Bennett 2004; Kranabetter *et al.* 2007; Näsholm *et al.* 2009). The variation in foliar $\delta^{15}N$ and %N were indicative of low N availability leading to a condition directly, via ECM, or indirectly, via soil biophysical processes, limits N bioavailability. Because internal ^{15}N fractionation in

black spruce roots and foliage was not consistent along the gradient future research should seek to clarify mechanisms. Finally, although ε factors based on $\delta^{15}N_{foliar}$ – $\delta^{15}N_{NH4}$ exhibited a positive relationship with resin [NH_4^+], we caution against widespread use of ε factors without detailed soil isotopic evaluations.

Table 3-1. Black spruce and fungal elemental contents and biomass across 31 central Alaskan forests.

PLANT & FUNGAL SUMMARY CHARACTERISTICS

	foliar %P	foliar %N	foliar $\delta^{15}N$	foliar $\delta^{13}C$	root $\delta^{15}N$	fungi $\delta^{15}N$	Hyphal ingrowth	Fungal PLFA PC1	Total biomass (ton ha^{-1})
mean	0.11	0.74	-7.32	-28.22	-4.97	6.51	1.79	-0.02	37.36
n	31	31	31	31	28	17	31	30	30
sd	0.03	0.13	1.49	0.6	1.5	2.54	0.47	0.42	27.69
range	0.08	0.47	6.55	2.53	5.98	9.48	1.66	1.73	97.36

Table 3-2. Soil fertility measurements made across 31 central Alaskan black spruce forests.

	DON org (µg N/g dry soil)	DON min (µg N/g dry soil)	NH4 res (ng N/g resin/day)	NO3 res (ng N/g resin/day)	DON- $\delta^{15}N$	NH4- $\delta^{15}N$	NO3- $\delta^{15}N$	Soil- $\delta^{15}N$	C:N org %	PO4 res (ng N/g resin/day)	CEC (meq)	soil moisture	pH	active layer depth (cm)
mean	301	54.3	1.07	0.13	6.49	2.83	0.78	0.42	38	12.5	18.3	26.9	5.58	63
n	31	29	31	31	31	31	7	31	31	31	30	30	30	30
sd	109	36	0.95	0.14	2.86	2.61	6.34	0.91	5.78	16.7	6.85	5.6	0.7	25.9
range	455	163	3.70	10.3	10.4	10.3	15.1	3.87	22.8	58.7	27.3	22.32	2.9	126

Table 3-3. High-ranking multiple regression models from 31 central Alaskan black spruce forest.

Model/Hypothesis	*df*	Δ_i	ω_i	$E_{i,j}$	R^2_{adj}
foliarN ~ DON-org	29	0.00	0.22	1.00	0.25
foliarN ~ DON-org + FunPC1	27	0.17	0.21	1.09	0.35
foliarN ~ DON-org + MinN-res	28	1.50	0.11	2.11	0.25
foliarN ~ DON-org + N-PC1	28	1.56	0.10	2.17	0.31
foliarN ~ DON-org + MinN-res + FunPC1	26	1.69	0.10	2.32	0.35
foliarP ~ P-PC1 + log(PO4-res)	27	0.00	0.30	1.00	0.60
foliarP ~ log(PO4-res)*Hyphae	27	0.00	0.30	0.98	0.59
foliarP ~ P-PC1	29	0.97	0.19	1.59	0.56
foliarδ^{15}N ~ δ^{15}N-PC1 + δ^{15}N-NH4	27	0.00	0.33	1.00	0.25
foliarδ^{15}N ~ δ^{15}N-PC1	28	0.07	0.32	1.04	0.21
foliarδ^{15}N ~ δ^{15}N-PC1*δ^{15}N-DON + δ^{15}N-NH4	25	2.03	0.12	2.76	0.30
Spruce biomass ~ δ^{15}N-PC1	28	0.00	0.59	1.00	0.33
Spruce biomass ~ δ^{15}N-PC1 + foliarN	27	1.26	0.32	1.88	0.33
FunPC1 ~ C:N	28	0.00	0.57	1.00	0.65
FunPC1 ~ C:N + DON-org	27	2.22	0.19	3.03	0.64
FunPC1 ~ C:N + CEC	27	2.29	0.18	3.14	0.64

df = degrees of freedom, Δ_i = bias-corrected Aikake Information Criterion, ω_i = model probability, $E_{i,j}$ = evidence ratio, R^2_{adj} = adjusted pearson's correlation coefficient . *foliarN* = % (g g^{-1}) N in black spruce foliage, *DON-org* = concentration of 2M KCL extractable dissolved organic N from soils in the middle of the growing season, *FunPC1* = PLFA-based principle component axis representing fungal biomass, *MinN-res* = mineral N measured from ion exchange resins, *N-PC1* = Principle component axis containing soil fertility metrics without extractable N concentrations included; *foliarP* = % (g g^{-1}) P in black spruce foliage, *P-PC1* = Principle component axis containing soil fertility metrics without extractable PO_4^- concentration included, *PO4-res* = resin extractable PO_4^- concentration, *Hyphae* = hyphal ingrowth biomass metric; *foliarδ^{15}N* = black spruce foliar δ^{15}N value, *δ^{15}N-PC1* = Principle component axis containing soil fertility metrics without soil N δ^{15}N values such as *δ^{15}N-NH4* or *δ^{15}N-DON*, *Spruce biomass* = standing aboveground biomass of black spruce estimated from allometric equations applied to stand basal areas, *C:N* = carbon to nitrogen ratio of organic soils, *CEC* = cation exchange capacity of organic soils.

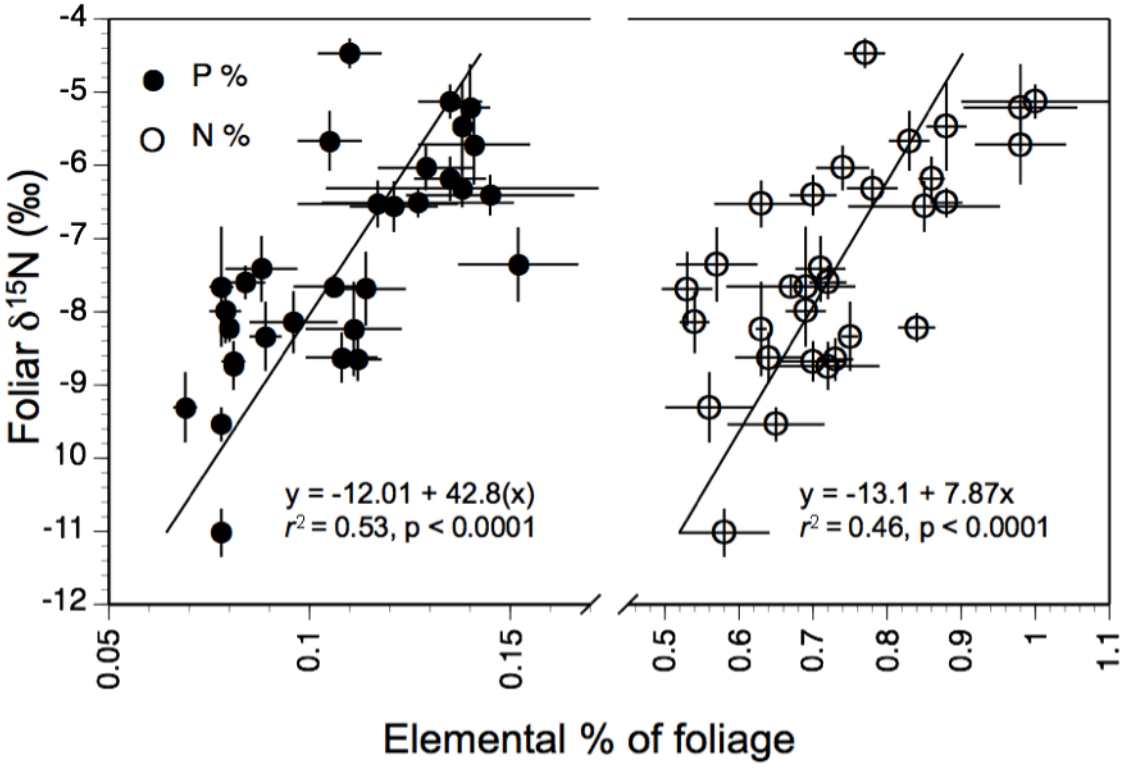

Figure 3-1. Average elemental content (%) of full sun foliage (N = 3) collected from black spruce (*Picea mariana*) trees in 31 plots in central Alaska. Phosphorus % (g^{-1}): R^2 = 0.53, P < 0.001; Nitrogen %: R^2 = 0.46, P < 0.001.

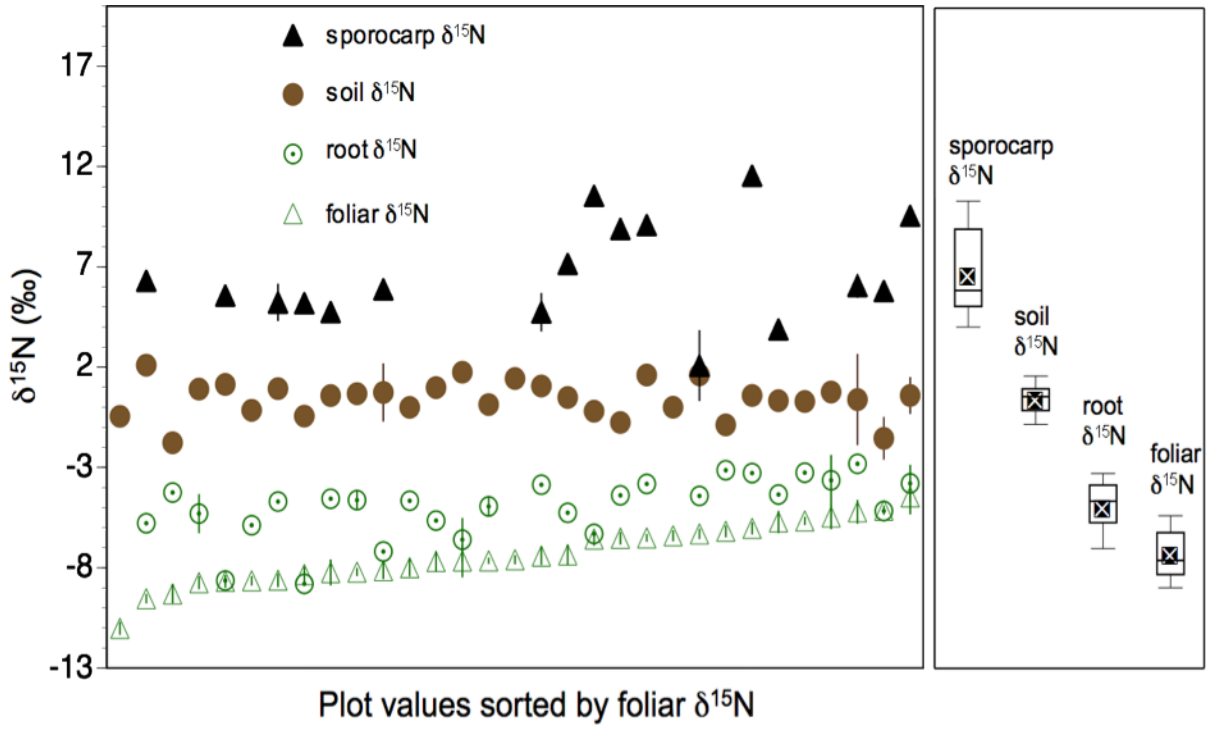

Figure 3-2. Average (± SE) black spruce full sun needle, fine root, bulk organic soil, and ectomycorrhizal sporocarp $\delta^{15}N$ values across 31 plots in central Alaska. Points are arbitrarily arrayed according to average foliar $\delta^{15}N$ values to illustrate the lack of strong covariance among components. Only foliar $\delta^{15}N$ was significantly correlated with root $\delta^{15}N$ (foliar^{15}N = -4.69 + 0.51$^{15}N_{root}$, R^2 = 0.30, P = 0.01). The slope for foliar $\delta^{15}N$ (0.16) is nearly twice that of both the ECM sporocarps and black spruce fine roots (0.09), although not statistically different. Across all plots, ecosystem components were different from one another (paired T-test, $P < 0.001$) and the mean isotopic differences between foliage and root, foliage and soil, and foliage and fungal $\delta^{15}N$ values were -2.35‰, -7.74‰, and -14.12‰, respectively.

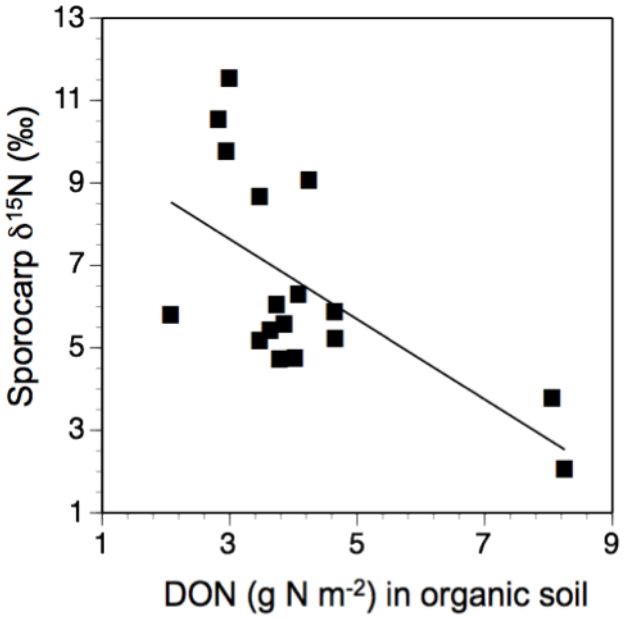

Figure 3-3. Relation among organic soil DON content and fungal sporocarp $\delta^{15}N$ values. R^2 = 0.39, $f(x) = -0.9635\chi + 10.5225$ with 15 degrees of freedom.

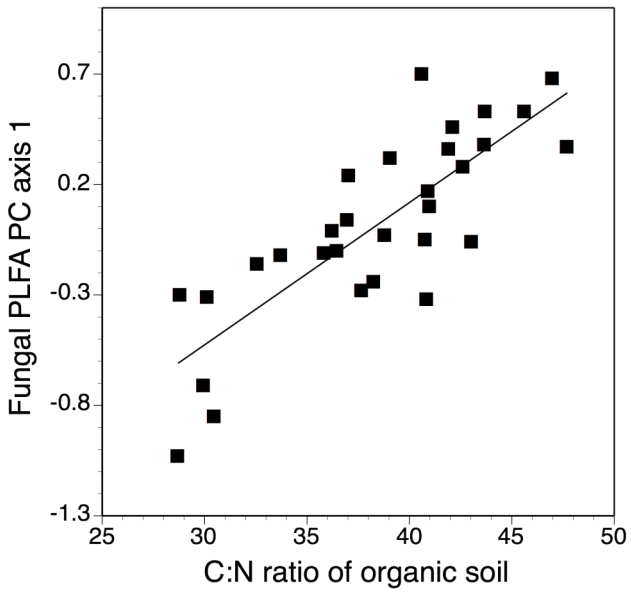

Figure 3-4. Relation among C:N ratios of the organic black spruce soils with the Phospholipid Fatty Acid (PLFA) based metrics of fungal biomass represented as the first Principle Component (PC) axis ($f(x) = -6.442E\text{-}2\chi + 2.458$; $R^2 = 0.65$). The PC axis values are arbitrary but correspond to increasing fungal biomass at higher C:N ratios.

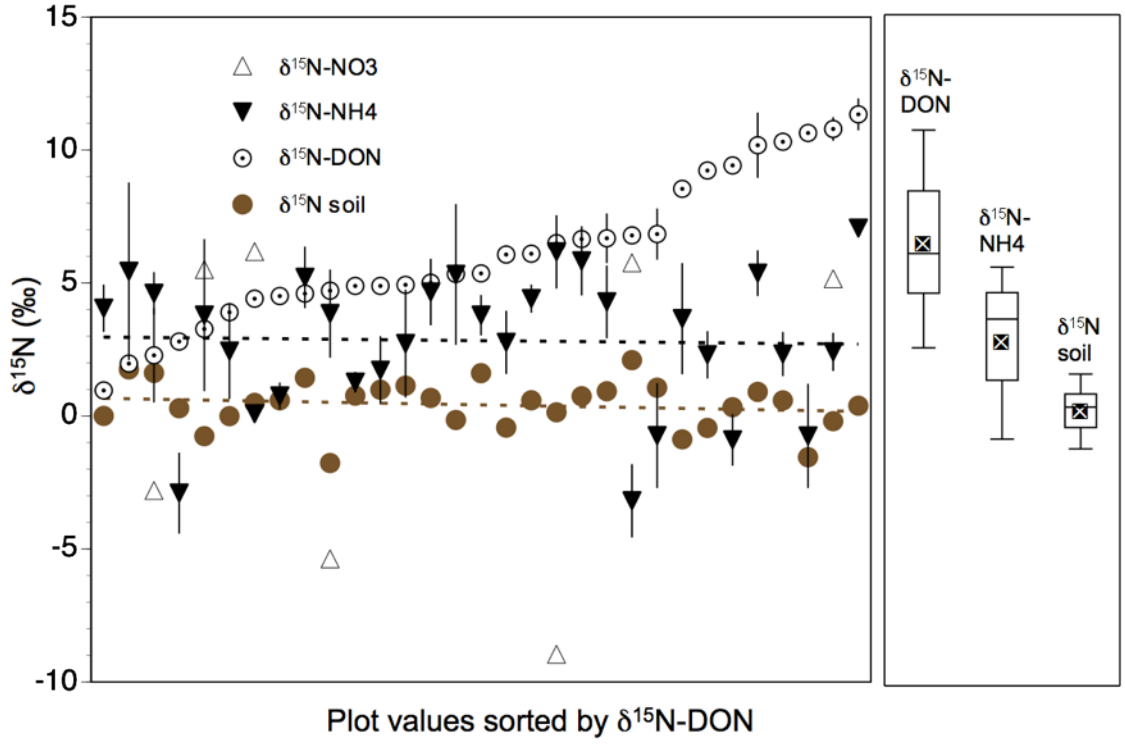

Figure 3-5. Mean (N = 3, ±SE) soil N $\delta^{15}N$ values from dissolved organic N (DON), ammonium (NH_4^+), and bulk organic soils across 31 plots in central Alaska. The N $\delta^{15}N$ values on are arbitrarily sorted by $\delta^{15}N_{DON}$ values to examine covariance among pools. The figure on the right is the mean (± SE) illustrating the differences among average pools (paired t-test, $P < 0.001$).

CHAPTER 4
DETECTING ALTERED NITROGEN CYCLES IN BLACK SPRUCE FOREST FOLLOWING FERTILZATION USING SOIL, PLANT, AND FUNGAL $\delta^{15}N$ VALUES

Abstract

Many northern forests are limited by nitrogen (N) availability, slight changes in which can have profound effects on elemental cycling and diversity of plants and microbes. Because climate-induced nutrient mineralization may increase N availability, experimental manipulation of soil nutrient availability offers insight into possible future ecosystem conditions. However, because N uptake occurs largely in an opaque soil medium it remains difficult to assess ecosystem N supply and cycling pathways without frequent measurements and the assembly of complete N budgets. Here we use detailed measurements of ecosystem stable isotope ratios of N ($\delta^{15}N$) to examine the form and pathways of N cycling following five years of NH_4NO_3 and/or super triple phosphate fertilization of mature black spruce forests in central Alaska. N fertilization reduced soil dissolved organic N (DON) content and when combined with P fertilizer, reduced black spruce dependency on ectomycorrhizal fungi by 36%. N fertilization also reduced the $\delta^{15}N$ values of fungal sporocarp and black spruce needles, but not soil N or roots, to approach that of the fertilizer. Surprisingly, P fertilization altered the soil N cycle in these stands as evidenced by increased NO_3^- concentrations and substantial ^{15}N enrichment of resin exchangeable soil NO_3^- relative to the control plots. Fractionation against ^{15}N during denitrification could account for the isotopic enrichment in the NO_3^- pool. Combined, our experimental approach illustrated that fertilization altered soil fertility, the pathways of N cycling, and the role of fungi in black spruce forest.

Introduction

Increased terrestrial N availability is becoming a global issue with impacts extending beyond industrialized regions of North Eastern North America and Northern Europe (Matson *et al.* 2002; Galloway *et al.* 2008). In most boreal forests anthropogenic deposition is less of a problem, but landscape modification and accelerated decomposition resulting from climatic warming can increase *in situ* N mineralization in otherwise severely N limited boreal ecosystems (Nadelhoffer *et al.* 1991; Mack *et al.* 2004; Hyvönen *et al.* 2007; Allison & Treseder 2008). As a result, productivity and ecosystem dynamics can be profoundly altered (Tamm 1990; Vitousek & Howarth 1991; Nordin *et al.* 2005; Elser *et al.* 2007). Therefore, determining baseline patterns in, and key integrative signals of, the N cycle are necessary to detect when natural N cycling conditions have been altered by climate, disturbance, or deposition. This research need is particularly strong in high latitude ecosystems where plants are heavily dependent on mycorrhizal fungi for access to slow cycling N pools.

Boreal ecosystems subjected to increased N availability may respond with greater carbon (C) fixation (Högberg *et al.* 2003), altered C allocation patterns (Nadelhoffer 2000; Vogel *et al.* 2008), accelerated nutrient cycling (Mack *et al.* 2004), and shifts in plant community diversity (Shaver *et al.* 2001; Mack *et al.* 2004). Enhanced N availability can alter microbial communities in uncertain ways, perhaps owing to the inherent difficulties in detecting changes (Wall *et al.* 2010). For instance, the biomass of ectomycorrhizal (ECM) and ericoid mycorrhizal fungi can increase or decrease in response to N addition (Clemmensen *et al.* 2006; Treseder 2008; Ishida & Nordin 2010; Janssens *et al.* 2010), and ECM, saprotrophic, and arbuscular mycorrhizal fungal community composition and diversity are typically altered or negatively affected

largely with unknown functional consequences (Wallenda & Kottke 1998; Egerton-Warburton & Allen 2000; Lilleskov & Bruns 2001; Peter *et al.* 2001; Avis *et al.* 2003; Nilsson *et al.* 2007). Changes in fungal species assemblages, for instance, may affect elemental cycling and plant community structure through direct and indirect mechanisms related to C allocation, residual litter quality, and enzymatic capacity of different fungal species (Lilleskov *et al.* 2002a; Lilleskov *et al.* 2002b; Lucas & Casper 2008; Högberg *et al.* 2010a). Furthermore, reduced belowground C allocation under increased nutrient availability may alter plant dependency on ECM-derived N (Treseder & Vitousek 2001; Nilsson & Wallander 2003), further exacerbating shifts in community diversity and function (van der Heijden *et al.* 2008).

Black spruce (*Picea mariana*) is the most prevalent tree in boreal forests of Alaska and Canada (Viereck & Johnston 1990). In these boreal forests N-limitation is severe because cold temperatures limit both growing season length and organic matter decomposition. These conditions, combined with highly recalcitrant moss and spruce litter, slow the N cycle to the point where the majority of labile N is in organic, rather than mineral form. The resulting dissolved organic N (DON) pool typically outweighs inorganic N by as much as 90% (Van Cleve & Yarie 1986; Jones & Kielland 2002; Yu *et al.* 2002). As growth requirements in Alaskan black spruce forest typically exceed annual N mineralization rates (Shaver & Chapin 1991; Shaver *et al.* 1992), a strong selective pressure for access to this DON is believed both common and necessary (Lipson & Nasholm 2001; Neff *et al.* 2003; Schimel & Bennett 2004).

Whereas, amino acid uptake occurs in numerous ecosystems, plant species, and mycorrhizal types (Wallenda & Read 1999; Näsholm *et al.* 2009; McFarland *et al.* 2010),

use of larger molecular weight organic N requires ECM fungal mediated depolymerization via exoenzyme (Talbot & Treseder 2010) or peroxidase production (Bödeker *et al.* 2009). Black spruce is extensively colonized by ECM fungi (Ruess *et al.* 2003) and both the autotrophic host and heterotrophic fungi can directly absorb a portion of the rapidly cycling amino acid pool (Jones & Kielland 2002; Allison *et al.* 2007; Kielland *et al.* 2007; Treseder *et al.* 2008). The ECM fungi, however, can also access a larger portion of organic N including proteins, peptides, and chitin (Abuzinadah & Read 1986; Abuzinadah & Read 1988; Lindahl & Taylor 2004; Read *et al.* 2004; Benjdia *et al.* 2006; Nygren *et al.* 2007). Furthermore, the observation that black spruce forests have the highest soil protease activities (e.g. the rate limiting step) recorded in central Alaskan boreal forest (Weintraub & Schimel 2005; Kielland *et al.* 2007) suggests that sustained productivity likely rests on continued access to the DON pool by ECM fungi.

Stable isotope ratios of N (^{15}N:^{14}N represented as $\delta^{15}N$) measured in different ecosystem N pools may offer clues for detecting and quantifying the dependency of black spruce on different forms and pathways of N absorption. These $\delta^{15}N$ values, however, often have multiple underlying causes that must be disentangled (Craine *et al.* 2009; Pardo & Nadelhoffer 2010). For instance, global mean annual temperature and precipitation correspond well with $\delta^{15}N$ patterns in plants, and to a lesser degree, in fungi and soils (Handley *et al.* 1999; Amundson *et al.* 2003; Mayor *et al.* 2009). At smaller landscape scales, however, the main controls over plant ^{15}N appear to be N status (measured as foliar %N), preferred N forms (DON, NH_4^+, NO_3^-), and the associated type or presence of mycorrhizae (arbuscular, ericoid, or ectomycorrhizal) (Michelsen *et al.* 1998; Högberg *et al.* 1999a; Miller & Bowman 2002; Hobbie & Hobbie

2006; Högberg *et al.* 2010b). Because plant $\delta^{15}N$ patterns are fundamentally influenced by isotope fractionation during microbial processing or losses of specific soil N forms as they 'leak' from an ecosystem (Gebauer & Schulze 1991; Nadelhoffer & Fry 1994; Nadelhoffer *et al.* 1996; Högberg 1997; Martinelli *et al.* 1999; Schuur & Matson 2001), measuring $\delta^{15}N$ values in distinct soil pools is necessary to definitively resolve underlying mechanisms (Houlton *et al.* 2006; Pardo *et al.* 2006; Templer *et al.* 2007; Kahmen *et al.* 2008).

Within a site, the types of soil N sources available may control plant foliar $\delta^{15}N$. Individual soil N moieties differ in $\delta^{15}N$ values because of ^{15}N discrimination during ammonification, nitrification, and denitrification within soil (Létolle 1980; Mariotti *et al.* 1981; Shearer & Kohl 1986). Assuming incomplete conversion, the expectations are that as the N cycle progresses, N moieties become successively ^{15}N depleted so that $\delta^{15}N_{DON} > \delta^{15}N_{NH4} > \delta^{15}N_{NO3} > \delta^{15}N_{N2}$. These differences, with some caveats, can then theoretically be traced to plants (Chapin *et al.* 1993; Pate *et al.* 1993; Nadelhoffer *et al.* 1996; Robinson 2001; Miller & Bowman 2002; Sah *et al.* 2006; Miller *et al.* 2007) once the methodological challenge of measuring $\delta^{15}N$ values of soil N moieties at field concentrations has been overcome. It is believed that plants may shift preference among N forms depending on factors such as forest disturbance (Pardo *et al.* 2002), climate induced changes to the most abundant N forms (Houlton *et al.* 2007), or in response to the application of fertilizer (Högberg 1997; Högberg *et al.* 2010b). Also, isotopic differences between mineral forms of N have been used to infer plant preferences of NH_4^+ versus NO_3^- in high latitude coniferous forest (Hobbie *et al.* 1998), temperate grasslands (Kahmen *et al.* 2008), and among co-occurring shrubs in tundra

and alpine ecosystems (McKane *et al.* 2002; Miller *et al.* 2007). Recently, the addition of DON to these analyses has more accurately extended the sources of N that can contribute to plant nutrition (Houlton *et al.* 2007; Takebayashi *et al.* 2010; Yano *et al.* 2010).

Further complicating the tracing of soil N to plants using $\delta^{15}N$ values is the possibility that N can be taken up directly from soil through black spruce roots or transferred with heavy ^{15}N fractionation through ECM fungi (Hobbie & Colpaert 2003). Furthermore, small isotope effects may be incurred during N translocation from roots to foliage (Robinson 2001). Each of these steps and pathways must therefore be quantified in order to accurately model N flux. The degree of ^{15}N-depletion in a given host plant is therefore expected to result from not only the sources of N, but also the proportional dependency on fungal derived N (Hobbie *et al.* 2000) followed by the translocation effects as N moves from root to needle. This sort of accounting has recently been demonstrated in tussock tundra (Hobbie & Hobbie 2006; Yano *et al.* 2010) and boreal forest undergoing forest succession (Hobbie *et al.* 2005) enabling the modeling of proportional N and C flux with associated ECM fungi (Hobbie & Hobbie 2008). Expanding upon these pioneering approaches with inclusion of $\delta^{15}N$ values from organic N pools illustrated that tussock tundra plants may depend on ericoid and ECM fungi for 30–60% of their total N requirements (Yano *et al.* 2010) and that black spruce may depend on ECM fungi for 86–99% N of their N uptake requirements (Mayor *et al.* Chapter 3).

Here we examine the N cycling patterns using detailed measurements of $\delta^{15}N$ in plants, fungi, and soil across 16 fertilized interior black spruce plots following 5 years of

mineral nutrient fertilization. The objective was to assess the utility of using $\delta^{15}N$ values to understand the form and pathway of N cycling in black spruce forest changes under elevated N and phosphorus (P) availability in a full-factorial manner. By combining detailed measurements of ecosystem $\delta^{15}N$ patterns with metrics of fungal biomass and growth, we explicitly sought to examine hypothesized relationships between soil mineral nutrients, ECM fungi, and plant $\delta^{15}N$ values.

Given that productivity of black spruce is severely N-limited, we hypothesized that N fertilization: causes plant $\delta^{15}N$ values to become less ^{15}N-depleted and ECM sporocarps to become less ^{15}N-enriched as a result of a general reduction in reliance on ECM-derived N by black spruce and increased use of the fertilizer N by both black spruce and associated fungi. We also hypothesized that fungal biomass declines in response to alleviation of plant N, but not P demands, leading to a reduction of below ground C allocation as 'payment' to the fungi for N. In contrast, we expected P fertilization to not influence plant or sporocarp $\delta^{15}N$ values or modeled dependency upon ECM fungi. With regards to soil N pools, we hypothesized that soil N $\delta^{15}N$ values to become less variable under N fertilization and to match that of the mineral fertilizer following 5 years of N additions. We expected P fertilization to have no influence on soil N $\delta^{15}N$ values in the absence of potential pH effects.

Methods

Site Description

Boreal forest is the second largest terrestrial biome in the world (Whittaker 1975) and black spruce (*Picea mariana* (Mill.) BSP) dominated forest is the most abundant forest type in boreal North America (Viereck & Johnston 1990), encompassing some

40% of interior Alaska alone (Vancleve & Dyrness 1983). Its success in the landscape is attributed to extreme freezing tolerance, the ability to grow in shallow permafrost soils with impeded drainage, as well as the ability to grow on well drained productive upland sites (Chapin III *et al.* 2006).

The experimental site was located approximately 15 km south of Delta Junction, AK, and consists of 16 plots arrayed in four blocks of four 10 × 10 m^2 plots. The black spruce forests are mature (~80 years old) and classified as dry nonacidic black spruce forest (Hollingsworth *et al.* 2006) featuring relatively xeric conditions with low rainfall (~300 mm yr^{-1} MAP), cold conditions (-2^oC MAT), and a relatively shallow organic layer (6.3 cm O horizon, Table 1) compared to many black spruce forests. The plant community overstory is dominated by black spruce but two of the blocks also contain a minor component of *Populus tremuloides*. The understory consists of minor components of *Betula glabra, Salix spp., Vaccinium vitis-idaea, V. uliginosum,* and *Rhododendron groendlandicum* ((Oeder) Kron & Judd) shrubs, along with 30-50% moss (mainly *Pleurozium schreberi* or *Hylocomium splendens*) or lichen (*Cladina, Cladonia*, and *Cetraria spp.*) cover (Treseder *et al.* 2004; Mack *et al.* 2008). Each treatment plot was fertilized in the early spring for 5 years prior to and including the 2007 summer field season when sampling for this study was conducted. In 2002, each plot received single broadcast doses of $NH_4^+NO_3^-$ (N), ortho-PO_4^- (P), both together (N+P), or none (control), annually at a level of 200, in year 1, or 100 kg ha^{-1} yr^{-1}, in subsequent years, per nutrient.

Field Sampling and Laboratory Analyses

Foliar $\delta^{15}N$, N, and P content (%) of needles were obtained from four mature black spruce trees in each plot. Five terminal full sun branches were collected from each tree and composited by tree at the peak of needle expansion during August 29-30, 2007. In addition, three ~2mm diameter roots were also carefully excavated from each of the same trees, composited, and refrigerated until outer secondary root tissue could be carefully removed approximately three weeks later. This step was necessary to prevent potential inclusion of fungal biomass in subsequent isotopic analyses (Högberg *et al.* 1996), although a minor component of ECM hyphae were likely present in the remaining root cortex. Needle and root tissue were dried at 60°C for 24 hrs, ground to a fine powder, and analyzed on a ThermoFinnigan continuous flow isotope ratio mass spectrometer coupled to a Costech elemental analyzer at the University of Florida. Stable isotope abundances are reported as:

$$\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000 \quad (4\text{-}1)$$

$R = {}^{15}N/{}^{14}N$ refers to the ratio of the sample and reference standard of atmospheric N_2. Run standard error rates were typically less than 0.2‰.

During the entire 2007 growing season, metrics of bioavailable NH_4^+, NO_3^-, and PO_4^- were obtained from field-incubated anion or cation exchange resins (Giblin *et al.* 1994). In addition, seven hyphal ingrowth mesh bags were also inserted throughout each plot at the organic and mineral soil interfaces to measure actively growing fungal mycelia (Wallander *et al.* 2001; Nilsson & Wallander 2003). The construction, deployment, and analyses of the resin and hyphal ingrowth bags are described in detail elsewhere (Mayor *et al.* Chapter 3).

Extractable soil N forms were obtained either from the exchange resins or from a 2 *M* KCl extract made at the height of the growing season, early August 2007. In each plot, three cores were extracted and composited from within three zones of each 10 × 10 m plot (12 total cores for each of 16 plots) with an SMS® volumetric slide hammer (4.2 cm diameter). The cores generally consisted of 5 cm of a mineral soil 'plug' with the remaining being compressed organic soil (~15 cm maximum). Green moss or lichens were removed, depth was recorded, and the horizons separated. Green moss was removed from the surface of each core prior to homogenization for subsampling. Each composited soil sample was stored on ice in the field or under refrigeration in the lab for approximately 24 hours prior to salt extraction, filtration, and freezing. Because 80% of black spruce roots are typically found in the organic horizons (Ruess *et al.* 2003) and the logistical limitations of high throughput denitrifier samples, only the organic soils were extracted for δ^{15}N values of the N moieties.

Total dissolved N was extracted from ~24 g (wet weight) subsamples in 2*M* KCL in nano-pure D.I. water, oxidized with persulfate/thermodigestion, and then coupled to the denitrifier method for δ^{15}N measurements as previously described in detail elsewhere (Knapp *et al.* 2005; Houlton *et al.* 2007), including specific modifications to the oxidation method (Chapter 3). The δ^{15}N value of the extractable dissolved organic N (DON) pool was then determined from the mass-weighted equation (4-2) below.

Sporocarp sample sizes varied from 12 to 29 across treatments with the fewest collected from the N+P and N treatments (*N* = 12 and 13, respectively), and the most in the P and control treatments (*N* = 22 and 29, respectively). Phospholipid fatty acid analyses (PLFA) of original frozen soil subsamples were performed on three 1-5 g (wet

weight) subsamples of the same organic soils as a metric of fungal biomass. This process involved an initial: lipid extraction, fractionation, and successive elution (Frostegård *et al.* 1991); followed by conversion of the methanol fraction into free methyl esters by mild alkaline methanolysis; and then analysis on a gas chromatograph with a flame ionization detector and a 50 m HP5 capillary column (Frostegård *et al.* 1993). The content of the specific PLFA 18:2ω6,9 was regarded as a proxy for total fungal biomass (Frostegård & Bååth 1996) and to mainly comprise ECM forming fungi in boreal forest (Allison *et al.* 2007; Lindahl *et al.* 2007; Taylor *et al.* 2010).

Statistical Analyses

Prior to ANOVA comparisons, parametric assumptions were assessed using Levene's test for homogeneity of variance and Shapiro-Wilk's test for normality (α = 0.05) using R® (2.9.2, The R Foundation for statistical computing 2009). Fertilizer treatment effects were analyzed using Dunnett's test of treatment against control or Tukey's HSD test on specific variables of interest. All ANOVA tests were performed with the inclusion of the experimental blocking effect in accordance with the factorial design of the experiment using JMP® 8.0.2 (2009 SAS Institute).

Mass Balance ^{15}N Mixing Models

The δ^{15}N value of black spruce represents the δ^{15}N value of all N sources taken up and the fractionation effects during their assimilation and transfer (Emmerton *et al.* 2001; Robinson 2001). To estimate proportional contribution and pathway of N flux to black spruce across treatments we used the following δ^{15}N based mass balance mixing models developed for arctic tundra ecosystems (Hobbie *et al.* 2009; Yano *et al.* 2010):

3 pool N source:

$$\delta^{15}N_{DON} = \delta^{15}N_{TDN} \bullet [TDN]_{KCl} - (\delta^{15}N_{NH4} \bullet [NH_4^+]_{KCl} + \delta^{15}N_{NO3} \bullet [NO_3^-]_{KCl})) / [DON]_{KCl} \quad (4\text{-}2)$$

$$\delta^{15}N_{available} = f_{DON} \bullet \delta^{15}N_{DON} + f_{NH4} \bullet \delta^{15}N_{NH4} + f_{NO3} \bullet \delta^{15}N_{NO3} \quad (4\text{-}3)$$

$$\delta^{15}N_{available} = (1 - Tr) \bullet \delta^{15}N_{fungi} + Tr \bullet \delta^{15}N_{transfer} \quad (4\text{-}4)$$

2 pool plant mixture:

$$\delta^{15}N_{root} = \delta^{15}N_{available} - \Delta f \bullet (1 - Tr) \bullet f \quad (4\text{-}5)$$

2 pool fungal mixture:

$$\delta^{15}N_{fungi} = \delta^{15}N_{available} + \Delta f \bullet Tr \quad (4\text{-}6)$$

where $\delta^{15}N_{DON}$ in equation (4-2) is derived from a mass weighted equation based on the original 2 *M* KCl concentration of N ions ($[N]_{KCl}$) and $\delta^{15}N$ values measured from resin extracted NH_4^+ and NO_3^- detailed elsewhere (Chapter 3). Equation (4-3) solves for the $\delta^{15}N$ value of the effective available N ($\delta^{15}N_{available}$) to plants and fungi based on proportionally weighted $\delta^{15}N$ values of the three soil source pools. Inclusion of the extractable DON pool as a potential N source enhances the biological realism of these N-based mixing models (Houlton *et al.* 2007; Yano *et al.* 2010) because DON can comprise greater than 90% of DON in high latitude ecosystems and inorganic N fluxes are typically unable to account for annual plant N requirements in these cold, high latitude ecosystems (Ruess *et al.* 1996; Neff *et al.* 2003; Valentine *et al.* 2006; Näsholm *et al.* 2009). In the remaining equations (4-4 to 4-6), Tr refers to the proportion of total fungal N that is transferred to host plants, $\delta^{15}N_{transfer}$ refers to the $\delta^{15}N$ value of the transfer compounds produced by ECM fungi, f refers to the proportion of plant N supplied by fungi, and Δf refers to the fractionation magnitude associated with transamination of soil N within ECM fungi (Hobbie & Hobbie 2006; Hobbie & Hobbie 2008).

We placed quantitative restraints on the source mixtures (DON, NH_4^+, NO_3^-) and pathways (ECM vs. direct uptake) of N flux to black spruce. Fractionation magnitudes associated with the transformation of soil N to δ^{15}N-depleted transfer compounds by ECM fungi (Δf) were estimated at Δ9 ±1‰ based on laboratory and field analyses as described in detail elsewhere (Hobbie & Hobbie 2008). Based on the extremely low NO_3^- concentrations in control plots (Table 4-1; 0.08–0.33% of 2*M* KCl extractable TDN was NO_3^-) and because ECM fungi strongly discriminating against NO_3^- under natural conditions (Rygiewicz *et al.* 1984; Clemmensen *et al.* 2008), we omitted $\delta^{15}N_{NO3}$ values from control plot mixing models, yet retained the possibility of a minor contribution (10%) in the P treatments because of a notable increase in soil [NO_3^-] adsorbed to resins relative to the control (Table 4-1; Figure 4-1). Plant, ECM sporocarp, and soil DON, NH_4^+, and NO_3^- δ^{15}N value end members were measured in replicate from each plot and means were used as soil N end members.

Results

Responses of Black Spruce Elemental Content to Fertilization

Nitrogen fertilization, alone and in combination with P, increased foliar N concentration by roughly 50% relative to the control (Figure 4-1 & 4-2a; $P < 0.001$, Dunnett's test) and caused ^{15}N enrichment (Figure 4-2b, $P = 0.018$ and $P = 0.005$ respectively, Dunnett's test). Similarly, P, alone and in combination with N, increased foliar P concentration relative to the control (Figure 4-2c; $P = 0.002$ and $P = 0.06$, respectively, Dunnett's test), while foliar δ^{15}N values were unaffected (Figure 4-1b). Root N concentration and δ^{15}N values were not affected by fertilization (Figure 4-2b). Foliar C content was higher and δ^{13}C values lower in the N treatment relative to the

control (data not shown), although the effects were marginal (P = 0.15, 0.055, respectively; Dunnett's tests).

Responses of Fungal Biomass and Sporocarp δ^{15}N to Fertilization

Fertilization did not alter fungal biomass as measured by PLFA 18:2ω6,9 (Figure 4-2d), but measured hyphal ingrowth was greater in N+P relative to N fertilized treatments (Figure 4-2e, P = 0.045, Tukey's HSD). Neither metric of fungal standing biomass or seasonal growth varied from the control (α = 0.10, Dunnett's test). Sporocarp δ^{15}N values were less ^{15}N enriched in the N treatment relative to the control (P = 0.046, Dunnett's test) and P fertilized plots (Figure 4-2f, P < 0.10).

Soil Fertility Metrics

Soil O horizon depth and bulk density of soils were uniform among treatments (n = 12 cores per plot; Table 4-1). Soil pH values were higher in the N treatments relative to the control (P = 0.02, Dunnett's test; Table 4-1). 2M KCl extractable TDN in both the control and P fertilized plots were dominated by DON (96%). In contrast, the extractable TDN pool in plots fertilized by N and N+P was only 13 and 14% DON, respectively, owing to the increases in resin exchangeable [NH_4^+] and [NO_3^-] and a halving of extractable [DON] relative to the control (Dunnett's test, P < 0.05; Table 4-1). The [NH_4^+] in the N treatment was more than twice as high as in the N+P treatment while [NO_3^-] was comparable (Table 4-1). Similarly, P fertilization increased resin exchangeable [PO_4^-] in soil solution (P < 0.001, Dunnett's test on Box-Cox Y transformed variable) although values were highly variable across plots (SE = 379 to 540; Table 4-1). Soil C:N was higher in the P treatment (P < 0.1) largely due to lower

soil N in the organic matter of the P relative to N+P treatment only (P = 0.11, Tukey's HSD).

Soil δ^{15}N Values

The δ^{15}N values of bulk soil organic matter did not vary across treatments despite five years of N addition (avgerage = 0.5 ± 0.14‰; data not shown). Similarly, average δ^{15}N values of the large salt extractable DON pool were unaltered by fertilization although both N and N+P fertilization increased the variability of $\delta^{15}N_{DON}$ values across plots (P = 0.006, Levene's test). The δ^{15}N value of resin extractable NH_4^+ became enriched in the N treatment relative to the control (P = 0.07, Dunnett's test) and in the N relative to the P treatments (P < 0.1, Tukey's HSD). This NH_4^+ enrichment in N treatment meant the relative ranking of isotope pools was shifted from the typical pattern in the control of $\delta^{15}N_{DON}$ values being more enriched than $\delta^{15}N_{NH4}$ (6.1 vs. 4.41‰, respectively; Table 4-2). The δ^{15}N value of resin extractable NO_3^- was enriched in P relative to all treatments (P < 0.001, Tukey's HSD) and the N+P treatment relative to the control (P = 0.07, Dunnett's test; Figure 4-3).

Mass Balance Mixing Results

Average solutions to mass balance mixing models, along with associated δ^{15}N end members, are reported on a plot-by-plot basis (Table 4-2). Black spruce was estimated to receive 68-94% (average = 82%) of total N from ECM fungi (denoted as f in equation 4-5) in control plots. The contribution of ECM to black spruce N nutrition was also estimated to increase along with greater estimated contributions of DON (f_{DON} in equation 4-3) in $\delta^{15}N_{available}$ calculations. Similarly, black spruce in both P and N fertilized stands were estimated to rely on ECM derived N for 74% and 76%,

respectively, of their N (Table 4-2), but only the P treatment followed the same pattern of greater f with greater f_{DON}. In contrast to the other treatments, black spruce trees in the N+P treatment were estimated to be less reliant upon ECM derived N relative to the control (46%, P = 0.03, Dunnett's test; Table 4-2).

Unlike the trends in control and P plots, no pattern with specific soil N sources and f estimates was observed. In the N+P treatment, two plots had increasing f values with greater contributions of soil NH_4^+ (f_{NH4}). Collectively, these findings are depicted graphically in a qualitative diagram that illustrates the complicated patterns in N acquisition and pathways of N flux across treatment types as informed by mass balance mixing models (Figure 4-4). A High proportional N flux through ECM fungi was estimated in both the control and P treatments (red arrow in Figure 4-4a) and this required a high dependency on DON (large black arrow in Figure 4-4a) to achieve mass balance. In contrast, there was overall a general lack of NO_3^- contributing to modeled N uptake. The N treatment, in contrast, shows a switching of soil N sources to include more NO_3^- at the expense of DON (Figure 4-4b) yet continued reliance upon ECM. Lastly, the N+P treatment illustrates a reduced reliance upon ECM-derived N (diminishing red arrow in Figure 4-4c) with a corresponding increase in direct uptake of soil N by black spruce. Estimated f values subsequently compared with multiple isotope and nutrient pools in the stands. The f values were negatively correlated with both NH_4^+ and DON based enrichment factors (R^2 = 0.43, P = 0.01, Figure 4-5a; and R^2 = 0.41, P = 0.01, Figure 4-5b), and foliar $\delta^{15}N_{foliage}$ (R^2 = 0.44, P = 0.01, Figure 4-5c), whereas they were positively correlated with extractable [DON] g^{-1} pools (R^2 = 0.75, P < 0.001, Figure 4-5d).

Despite the averaged estimated f values being well constrained across plots (average *SE* = 0.025), mass balance was not achievable in two plots because subsequent mixture values did not fall within the available end-member sources ("n/a" in Table 4-2). In addition, in order to produce sensible results in one plot, a higher proportion of NO_3^- (0.7) was required despite a priori assumptions limiting proportional NO_3^- contribution to 0.5. Valid solutions in these plots were impaired by either particularly depleted sporocarp $\delta^{15}N$ or enriched $\delta^{15}N_{NH4}$ end member values (Plot *14-N* and *12-P*, respectively; Table 4-2).

For the control and P treatments, root $\delta^{15}N$ values were used as plant end members in mass balance equations instead of foliage because this permitted accounting of a variable internal plant fractionation demonstrated in black spruce of central Alaska (Chapter 3). In contrast, in the N and N+P treatments, only foliage responded to fertilization implying roots $\delta^{15}N$ values were poor integrators of plant N sources. Because of this lack of root response foliar $\delta^{15}N$ values were deemed better end member values for N fertilized plots. Furthermore, using these two different end member values for black spruce was necessary to achieve mass balance and was justified by the belief that the roots measured were not representative of the fertilizer N acquisition for the following reasons: 1) internal assimilation and/or translocation of N from roots to foliage produce negative fractionations not positive as would be necessary here (Robinson 2001; Kolb & Evans 2002); 2) root N content and $\delta^{15}N$ values were not altered by N fertilization, yet foliage was, suggesting no increased N allocation to roots; and, 3) a presumed low N demand in roots because non 1st order ECM roots (e.g. >2 mm) have long, yet poorly constrained, turnover times likely exceeding one year (Ruess

et al. 2006) and are less active physiologically than the smallest diameter fine roots (Pregitzer 2002).

When solving mass balance mixing models proportional mixtures of soil N were iteratively adjusted for each plot under the following conditions: (a) the proportion of black spruce derived from ECM fungi (f) could not exceed 100%; (b) mixtures of the different N forms occurred at 10% increments, allowing a maximum of 10 different values from 0 to 100%; (c) proportional mixtures in the control and P plots were constrained to a 2-source mixing model with a priori DON proportions constrained between 20-70%; and, (d) for the eight plots receiving N fertilizer, we adjusted the range of 3 source contributions to include low (0%) to high (50%) proportional DON concentrations with the remainder equally distributed across the mineral N fertilizer with NO_3^- contributions restrained to equal or lesser proportional contributions than NH_4^+. These conditional rules were justified because NO_3^- availabilities were extremely low under natural conditions (Table 4-1) and because partitioning of soil DON into bioavailable components from other studies indicate 100% of the DON pool is not available yet a minimum of 20% is entirely feasible based on multiple lines of evidence (Jones *et al.* 2005b; Yano *et al.* 2010). In P fertilized plots, where $\delta^{15}N_{NO3}$ contributions were permitted at 10%, inclusion of NO_3^- only acted to increase dependency on ECM but was not necessary to achieve mass balance. Given that, models were mathematically underdetermined, the results are presented as ranges of likely mixtures.

Discussion

Effects of Fertilization on Soil Fertility and Soil $\delta^{15}N$ Values

Fertilization with mineral N, both with and without P, caused a decline in DON but no change in average $\delta^{15}N_{DON}$ or bulk soil $\delta^{15}N$ values (Table 4-2, Figure 4-3). Mineralization of NH_4^+ from DON has been shown to cause large shifts in $\delta^{15}N$ values (e.g. 17‰) in single compound studies in the lab, particularly during phenylalanine ammonia-lyase activity (Werner & Schmidt 2002). However, many isotope studies in terrestrial ecosystems indicate that N isotope fractionation during mineralization is relatively small (Amundson *et al.* 2003; Evans 2007; Hobbie & Ouimette 2009). The similar $\delta^{15}N$ values measured from DON and NH_4^+ pools in control, P, and N+P fertilization treatments support the later interpretation. It was surprising that fertilization with N alone caused no difference among average soil $\delta^{15}N_{DON}$ and bulk soil $\delta^{15}N$ values, relative to the control, given the plots had received cumulatively 600 kg ha^{-1} of N fertilizer over the course of the 5-year experiment. Only the N treatment led to ^{15}N enrichment of the NH_4^+ pool ($P < 0.1$; Figure 4-3). Although other N addition experiments of similar magnitude and duration have caused isotopic changes in soil total N (Pardo *et al.* 2007), a general lack of changes here may indicate long turnover times of the soil organic N resulting from climatic extremes limiting microbial activity (Van Cleve & Alexander 1981). The variability of the DON pool, in contrast, suggests that mineralization of some fraction of the DON pool did occur during the preceding 5 years.

Because the reduction in extractable DON under N and N+P fertilization (Table 4-1) also corresponded with increased variance of $\delta^{15}N_{DON}$ values ($P = 0.03$, Levene's

test; Figure 4-3), it is likely these two observations are mechanistically related. For instance, the decline in DON suggests reduced production and/or accelerated decomposition under substantially higher mineral N availability. Reduced DON production could result from the release of mineral N requirements for fungal growth, thereby limiting the need for exoenzyme production. N fertilization has been shown to decrease lignolytic activity (Neff *et al.* 2002; Waldrop & Zak 2006; Lucas & Casper 2008). In contrast, N fertilization increases proteolytic enzyme activity in fungi (Lucas & Casper 2008). Reduced lignolytic activity combined with increased proteolytic activity would cause the more labile proteins in the DON pool to decline rapidly over the course of the study and recalcitrant complexes, including bulk soil N, to be retained longer. Following this line of reasoning, the remaining recalcitrant DON pool would be comprised of a greater proportion of recalcitrant compounds with correspondingly distinct $\delta^{15}N$ values and bulk soil N would remain relatively unaffected. The invariance in bulk soil $\delta^{15}N$ values clearly illustrates the isotopic disconnect from plant available N in this ecosystem.

Surprisingly, P additions also influenced the N cycle in these otherwise N-limited boreal forests. When N fertilization was combined with P, $\delta^{15}N_{NO3}$ values became ^{15}N enriched relative to the control (Figure 4-3). However, P addition alone caused an even larger ^{15}N enrichment of the smaller residual NO_3^- pool. The P treatment led to a 60-fold increase in adsorbed [NO_3^-] and 14 fold increase in adsorbed [NH_4^+] relative to the control, although only [NO_3^-] was significantly higher given high variability in [NH_4^+] (Figure 4-3). In contrast, 2*M* KCl extractions taken at the height of the growing season and used to extract total dissolved N, did not indicate any increase in standing [NO_3^-] or

[NH_4^+] pools (data not shown). Combined, these findings suggest that P fertilization accelerated the rate of mineral N cycling, but not necessarily the standing labile N pool size.

Accelerated N cycling caused by P fertilization could result from multiple processes. For instance, a stimulating influence on nitrifying bacteria could be the cause, either directly through elevated P bioavailability (Purchase 1974; Mahendrappa & Salonius 1982), or indirectly via pH influences on enzyme activity (Persson & Wiren 1995; Sinsabaugh *et al.* 2008), or through stimulation of mineralization rates and subsequent relief of NH_4^+ substrate limitations to nitrifiers (Munson & Timmer 1991). As both pH and [NH_4^+] were statistically indistinguishable in the P fertilized plot from that in the control (Table 4-1), the hypothesis that direct P fertilization of nitrifying bacteria is most plausible given the evidence available. Furthermore, a higher C:N ratio in the P fertilized treatments (Table 4-1) also indicates an accelerated N cycle, where soil N (but not soil C) was gradually reduced in response to P fertilization of decomposers.

Alteration of the N cycle from P addition in boreal forest ecosystems is not well understood. Most studies in N-limited ecosystems have instead examined or assumed that N fertilization, but not P fertilization, benefits plant growth and that microbial growth responds to alterations of below ground C allocation by these plants (Högberg *et al.* 2003; Janssens *et al.* 2010). However, bioavailability of both organic and inorganic P in boreal soils can be limited by aluminum-iron complexes in organic material (Giesler *et al.* 2004) and N mineralization rates in other ecosystems have been observed to accelerate following P additions (Munevar & Wollum 1977; Ross & Bridger 1978; Haynes & Swift 1988; Sinsabaugh *et al.* 1993). Of the abundance of N-fertilization

studies in spruce forest, only a few factorial P-addition studies were found, and of these, only one of three measured increased labile soil NO_3^- concentrations and mineralization rates following PO_4^- addition (Mahendrappa & Salonius 1982). This study occurred in a 55-60 year old black spruce stand from New Brunswick, Canada and the fertilizer was also super triple phosphate administered at comparable levels to our study. In contrast, two other P fertilization experiments, one in a Norway spruce plantation in Denmark, the other in a Sitka spruce plantation in North Wales, UK, measured a decline in mineral N following P fertilization (Stevens *et al.* 1993; Vesterdal & Raulund-Rasmussen 2002). Determining the mechanism for these findings is made difficult, however, because P fertilization occurred together with other mineral nutrients, although increased N-demand of spruce following release of spruce P-limitation is plausible.

Apart from increasing soil NO_3^- concentrations, P additions also induced a strong isotopic influence over $\delta^{15}N_{NO3}$ values, causing 17‰ enrichment relative to the control. Fractionation effects of this direction and magnitude likely reflect losses of $^{14}N\text{-}NO_3^-$ from the system. In Delta Junction soils, NO_3^- losses to leaching are unlikely given the extremely low rainfall (290 mm yr^{-1}) limiting the leaching potential even during the June, July, and August growing season which receives 65% of MAP (Mack *et al.* 2008). Several lines of evidence suggest gaseous losses lead to the observed NO_3^- enrichment instead. First, denitrification has been shown to discriminate against ^{15}N by as much as -27.2 ±2.8‰, calculated as: $\varepsilon_{denitrification} = \delta^{15}N_{product} - \delta^{15}N_{substrate}$ (Pörtl *et al.* 2007) and gaseous NO_x and N_2O losses during nitrification can be even higher, estimated at -34.7 ±2.5‰ (Mariotti *et al.* 1981; Hobbie & Ouimette 2009). The realized degree of enrichment, however, depends on the proportion of substrate consumed. The

second line of evidence comes from the $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios of the remaining NO_3^- pool, a pattern demonstrated in forest groundwater where denitrification contributes appreciably to N losses (Aravena & Robertson 1998; Mengis *et al.* 1999; Houlton *et al.* 2006). As expected if denitrification caused the observed enrichment, $\delta^{15}N$ and $\delta^{18}O$ values of NO_3^- were strongly correlated only within the P treatment (R^2 = 0.78, N = 4, P = 0.11; data not shown). A strong correlation between the particular denitrifier gene *nir*K and available P in a Central European spruce forest also suggest nutrient sensitivity of denitrifying bacteria (Bárta *et al.* 2010). Denitrification in many high latitude N limited systems is thought to be quantitatively unimportant owing to the low NO_3^- levels (Stehfest & Bouwman 2006; Hobbie & Ouimette 2009) but the mechanisms governing gaseous N losses (e.g. N_2O and N_2) in arctic soils are generally not well understood (Chapin 1996; Wolf & Brumme 2003; Siciliano *et al.* 2009).

Effects of Fertilization on Plant %N, %P, and $\delta^{15}N$

Nitrogen addition caused a doubling of black spruce foliar N concentration and, as a result of the uptake of N fertilizer, approximately 3‰ ^{15}N enrichment of foliage (Figure 4-1 & 4-2b). Beginning with some of the first field applications of $\delta^{15}N$ measurements in agricultural systems it has been known that fertilization with N causes actively growing plant tissue to more closely approximate the isotopic value of the fertilizer, typically near the atmospheric standard of 0 ±2‰ (Kohl *et al.* 1973; Shearer & Legg 1975; Vitoria *et al.* 2004). Given black spruce is one of the most ^{15}N depleted trees globally (Craine *et al.* 2009), absorption of near 0‰ fertilizer N would expectedly lead to relative ^{15}N enrichment. Whereas short duration fertilization experiments have documented this expected trend in temperate (Magill *et al.* 1997; Davis *et al.* 2004) and

boreal forest (Eriksson *et al.* 1996; Bergholm *et al.* 2007), prolonged fertilization can also lead to large losses of soil N and successive ^{15}N enrichment of residual soil N pools well beyond that of the fertilizer (Pardo *et al.* 2007). Under these conditions, plant tissue is expected to be substantially more ^{15}N enriched as a result of plant absorption of the resultant ^{15}N enriched residual N pool as seen in red spruce in Vermont (Pardo *et al*., 1998; McNulty *et al*., 2005) and in pine plantations in temperate and boreal forests (Hogberg 1991; Choi *et al.* 2005; Högberg *et al.* 2010b).

Unlike black spruce foliage, fine root N concentration and $\delta^{15}N$ values were surprisingly unaffected by fertilization (Figure 4-2b). Whereas fertilization can increase above-ground production (Mack *et al.* 2004), root production was unaffected by fertilization in Alaskan tundra (Nadelhoffer *et al.* 2002) and can decline in other ecosystems. For instance, six years of N fertilization in a Norwegian spruce plantation led to a 69% relative increase in total N pools but a 30% decrease in fine root N pools (Bergholm *et al.* 2007). This preferred allocation of new fertilizer N to foliage, combined with old root N reallocation to new roots could account for the observed patterns (Kolb & Evans 2002). Because NO_3^- can be biochemically assimilated by either the root or shoot tissue, and NH_4^+ can only be assimilated by the root (Andrews 1986), it has been hypothesized that intra-plant variation in $\delta^{15}N$ can result from differential assimilation of NO_3^- (Evans 2001; Kolb & Evans 2002). However, the isotopic effects of such differences could not cause the patterns we observed in black spruce. For instance, nitrate reductase activity discriminates against ^{15}N, so root assimilated fertilizer NO_3^- would actually cause relative enrichment of root tissue concurrent with the translocation of unassimilated isotopically light NO_3^- from root to shoot. Whereas this mechanism

could explain foliar patterns, it would not explain the lack of a root response observed here. Instead, it appears that fertilizer N is preferentially allocated only to growing needles, leaving old root N as the only retained N source to new roots. This discovery reinforces the idea that all plant tissue $\delta^{15}N$ values do not necessarily reflect N source $\delta^{15}N$ values.

Black spruce P concentrations in needles also nearly doubled in response to P fertilization, which indicates either substantial excess consumption or a stoichiometric relationship among N:P ratios (Ågren 2008). N:P ratios of needles were higher in N fertilized stands (17) compared to all other treatments and those in N+P treatments were higher than just P alone (9 versus 5; $P < 0.05$, Tukey's HSD). The control and P treatment N:P ratios were statistically indistinguishable. Relative ranking of treatment N:P ratios was therefore: N (17) > N+P (9) > C (7) > P (5). Because C and P treatments were equivalent, yet P increased more in the N+P relative to P treatments, black spruce foliar elements may indicate achievement of a stoichiometric balance and co-limiting growth conditions under N+P fertilization (Elser *et al.* 2007).

Preliminary above ground plant biomass and N tissue concentration data from the fertilized plots don't indicate any changes in the plant community apart from mosses. Mosses had higher N concentrations under both N and N+P fertilization ($P \leq 0.01$, Dunnett's test), yet N fertilization reduced moss biomass overall ($P = 0.07$, Tukey's HSD; M.C. Mack, unpublished data). No changes in either black spruce biomass or %N were seen in P-fertilized plots according to preliminary analyses of stem diameter growth data (M.C. Mack, unpublished data).

Effects of Fertilization on Fungal Biomass, Ingrowth, and $\delta^{15}N$

Fungal biomass and hyphal ingrowth trended to decrease in N fertilized plots although not significantly so relative to the control (Figure 4-2d,e). Declining biomass of mycorrhizal fungi is expected under increasing N availability as previously demonstrated in natural forest ecosystems (Nilsson *et al.* 2005; Treseder 2008; Wallander *et al.* 2009) and after single N fertilization events (Högberg *et al.* 2010a). Reduced ECM fungal biomass following fertilization is thought to result from a reduction in below ground C allocation to ECM fungi in response to a decline in mineral nutrient requirements by the autotrophic host plant (Högberg *et al.* 2003; Hobbie 2006; Högberg *et al.* 2010a). The lack of declines in fungal biomass or ingrowth in our study may be due to low statistical power resulting from a logistically limited number of PLFA analyses and a short incubation period of the ingrowth bags.

Although microbes are generally considered C limited, mycorrhizae are unique in that they have access to relatively abundant C supplies from their autotrophic hosts. Therefore, stimulation of mycorrhizal ingrowth in response to N+P fertilization may indicate mineral nutrient limitations of the exploring hyphae (Clemmensen *et al.* 2006; Treseder *et al.* 2008). Apart from standing biomass and ingrowth responses, abrupt increases in N bioavailability can also reduce the diversity of ECM fungi (Lilleskov *et al.* 2001; Lilleskov *et al.* 2002a; Frey *et al.* 2004). Although ECM diversity was not measured in our plots, a decline in saprotrophic species in our N fertilized plots was detected (Allison *et al.* 2007). Declining or altered belowground diversity may have unknown but potentially negative consequences to soil C stabilization and mineral nutrient cycling that deserves more attention, especially in light of our changing climate (Courty *et al.* 2010).

Similar to the black spruce autotrophic hosts, N fertilization caused sporocarp $\delta^{15}N$ values to shift down toward the applied N fertilizer (Figure 4-2f). The near 50% loss of ^{15}N enrichment under N fertilization suggests fungal use of the N fertilizer. Alternatively, declining sporocarp $\delta^{15}N$ values could also represent reduced processing or retention of ^{15}N during the transfer of ^{15}N-depleted N to host plants. However, this later explanation is unlikely given that no similar patterns were found in the N+P treatments where proportional N transfer by ECM fungi was modeled to be lower (see next section). As ECM fungal genera differ in their levels of ^{15}N enrichment (Trudell *et al.* 2004; Hobbie & Agerer 2009), changes to the ECM fungal community under N fertilization (Lilleskov *et al.* 2001) could have also contributed to the pattern of ^{15}N depleted ECM sporocarps in the N treatment.

Fertilization Induced Decline in Black Spruce Dependency on ECM

Plot specific estimates of black spruce dependency on ECM derived N (f) were, on average, lower in the N+P treatment relative only to the control (Table 4-2; Tukey's HSD, $P = 0.027$). Why was a similar decline in dependency on ECM-derived N not also found in N treatments as well (Treseder & Vitousek 2001)? One possibility is that increased P demands in the N treatment causes continued N delivery resulting from the inability of ECM fungi to selectively deliver only one mineral nutrient at a time. This hypothesis can account for the observation that ECM-dependency declines only when both N and P fertilization are combined. Under this interpretation, sporocarp $\delta^{15}N$ values become depleted under continued N fertilization because of mineral N dilution of otherwise enriched $\delta^{15}N$ values, not declining ^{15}N retention and transfer of N. Why there were no changes in sporocarp $\delta^{15}N$ values following N+P fertilization, however, is

unknown and may indicate an additional mechanism for ECM sporocarp ^{15}N enrichment.

To understand why only the combination of N+P fertilizers led to a decline in f we regressed it against several other potentially meaningful site variables (Figure 4-5). Examining the relationships among variables that were either directly or indirectly input into the mass balance mixing models to solve for f allows for an examination of relative relationships among variables. We found that the N+P treatment plots were among those with: the smallest isotopic differences between black spruce foliage and resin exchangeable NH_4^+ and DON (Figure 4-5a,b); the most enriched black spruce needle $\delta^{15}N$ values (Figure 4-5c); and, the lowest extractable [DON] pools (Figure 4-5d). There were no relationships found with estimates of f and bulk soil $\delta^{15}N$, soil C:N ratios, PLFA-based fungal biomass, or soil DON, NH_4^+, and NO_3^- concentrations.

What could these correlations indicate about changes to the functioning of these forests under fertilization? Declining foliar $\delta^{15}N$ values in the same tree species along successional chronosequences have been hypothesized to represent declining dependency on ECM fungi for N (Hobbie *et al.* 2000; Hobbie *et al.* 2005). Here foliar $\delta^{15}N$ values were positively correlated with f estimates, suggesting declining dependency on ECM-derived N is reflected in foliar $\delta^{15}N$ values (Figure 4-5c). However, foliar $\delta^{15}N$ values were uncorrelated with f estimates from 30 additional black spruce sites spanning a diversity of soil fertilities in central Alaska (Chapter 3). As mentioned previously, the same pattern could result from ^{15}N enrichment resulting from use of the applied mineral N fertilizer (Figure 4-2b). This issue was recently addressed in a Swedish pine forest while assessing the return of ECM dependency following

abandonment of N fertilization 6 years prior (Högberg *et al.* 2010b). These authors suggested it was not the fertilizer signature that led to subsequent depletion of *Pinus sylvestris* needle $\delta^{15}N$ values, but rather a return of ^{15}N retention by associated ECM fungi during their assimilation and deliver of soil N to their autotrophic hosts. Their determination was based largely on indirect evidence that their fertilizer was ^{15}N depleted relative to endogenous N sources and that ECM biomass was correlated with foliar $\delta^{15}N$ (Högberg *et al.* 2010b).

Declining ε_{DON} and ε_{NH4} with low f in part reflects the foliar $\delta^{15}N$ values used to calculate these metrics but the smaller differences at low f values also could indicate a reduction in isotopic discrimination resulting from ECM delivery of N. A relationship with f and $\delta^{15}N_{NH4}$ (R^2_{adj} = 0.16, P = 0.01, y = 0.85 + 0.017χ), ECM sporocarp, and root $\delta^{15}N$ values (R^2_{adj} = 0.39, P = 0.004, y = 1.07 - 0.025χ; R^2_{adj} = 0.16, P = 0.02, y = 0.74 - 0.03χ, respectively) was also observed across a black spruce soil fertility gradient (Chapter 3) suggesting $\delta^{15}N_{NH4}$ is a useful integrator of ECM activity when compared with host plant $\delta^{15}N$ values in boreal spruce forest. We must use caution when over interpreting these results because these values were involved, either directly or indirectly, in isotope mixing models used to estimate f, and could be interpreted as auto-correlated.

Lastly, the correlation of low f N+P plots with low soil [DON] and highly variable $\delta^{15}N_{DON}$ values suggests that as ECM fungi are relied upon less for N (e.g. low f), they also presumably use less of the now depleted and more recalcitrant DON pool. Oddly, no pattern was observed with proportional contributions of DON in the four N+P plot mixing model solutions (perhaps due to the variable $\delta^{15}N_{DON}$ values) but this pattern

was seen in both the control and the P treatments as well as in the majority of 30 mixing model solutions in another study of black spruce $\delta^{15}N$ patterns (Chapter 3).

Conclusion and Ecosystem Implications

Fertilization with N caused no changes in $\delta^{15}N$ values of bulk soil or DON pools but did cause black spruce foliar $\delta^{15}N$ values to become more enriched and foliage to contain twice as much N g^{-1} leaf. N fertilization also caused fungal biomass and ingrowth to decline slightly, as expected, while also causing ECM sporocarp $\delta^{15}N$ values to decline, but these trends were not observed in the N+P treatment. N fertilization, with or without P, also caused a reduction in [DON]. P fertilization similarly doubled foliar P concentration and had surprising influence on the N cycle, causing a spike in exchangeable soil [NO_3^-] and marked ^{15}N enrichment of the same pool likely caused by increased gaseous losses. When plots were fertilized with both N and P soil [DON] also declined nearly 50% as in the N fertilized plots, presumably due to alterations to enzyme production by the decomposer community. Furthermore, N+P fertilization caused shifts among both the form and pathway of N cycling through the soil-fungus-plant continuum with significant declines in the dependency for N delivery by ECM fungi.

Collectively, experimental alterations to soil fertility led to higher foliar N and P concentrations that will likely increase N-cycling rates (Magill *et al.* 1997), a reduction in labile [DON], and presumably reduced C allocation to ECM fungi. These can effects can have important consequences for soil carbon storage in boreal forests (Neff *et al.* 2002; Mack *et al.* 2004). Lastly, we demonstrated that ecosystem $\delta^{15}N$ values contain interpretable signals regarding forest N cycling even with complex sources of ^{15}N fractionation associated with ECM fungi.

Table 4-1. Soil characteristics across black spruce fertilization treatments ± *SE*. Each treatment corresponds to four plots. C = control, N = nitrogen addition, P = phosphorus addition. ** = difference from control ($P < 0.05$, Dunnett's test), * = $P < 0.1$.

Treatment	O horizon depth (cm)	Bulk density ($g\ cm^{-3}$)	DON ($ug\ g^{-1}$ soil)	DON ($g\ N\ m^{-2}$)	NH_4 ($\mu g\ g^{-1}$ resin day^{-1})	NO_3^- ($\mu g\ g^{-1}$ resin day^{-1})	PO_4^- ($\mu g\ g^{-1}$ resin day^{-1})	C:N	pH (H_2O)
C	5.80 ± 0.52	0.16 ± 0.03	313.97 ± 35.39	2.95 ± 0.61	1.08 ± 0.49	0.08 ± 0.01	3.86 ± 1.86	24.96 ± 1.56	4.75 ± 0.06
N	6.31 ± 0.60	0.12 ± 0.02	148.86 ± 09.97 **	1.09 ± 0.20 **	382.32 ± 96.66 **	399.30 ± 130.24 *	3.19 ± 1.33	24.77 ± 0.94	4.99 ± 0.06 **
N+P	7.09 ± 1.08	0.14 ± 0.03	135.94 ± 40.81 **	1.23 ± 0.37 **	168.11 ± 68.23 *	463.69 ± 151.10 **	839.05 ± 539.66	25.36 ± 0.60	4.87 ± 0.06
P	5.97 ± 0.35	0.15 ± 0.01	279.89 ± 19.70	2.42 ± 0.07	14.31 ± 7.95	67.43 ± 29.15	1008.14 ± 378.60	29.15 ± 1.28 *	4.74 ± 0.08

Table 4-2. Mass balance mixing results to estimate the proportional dependence of black spruce on ECM-derived N and the average (±SE) end member sources of N used across fertilization treatments.

Block	Plot	Treatment	f of ECM N	$\delta^{15}N_{DON}$	$\delta^{15}N_{NH4}$	$\delta^{15}N_{NO3}$	$\delta^{15}N_{Plant}$	$\delta^{15}N_{fungi}$
1	1C	Control	0.68 ± 0.23	7.29	3.77	0.18	-2.43	11.55
2	5C	Control	0.90 ± 0.00	4.73	5.19	-2.73	-5.18	11.31
3	10C	Control	0.94 ± 0.02	6.99	3.81	-1.16	-2.6	7.85
4	13C	Control	0.78 ± 0.03	5.4	4.87	-6.41	-0.68	7.42
		avg. (SE)	*0.82*	*6.10 (0.62)*	*4.41 (0.36)*	*-2.53 (1.42)*		
1	2N	Nitrogen	0.91 ± 0.04	5.41	5.88	1.12	-3.38	5.99
2	6N	Nitrogen	0.53 ± 0.04	-3.22	7.69	0.6	-1.29	6.97
3	9N	Nitrogen	0.85 ± 0.04	9.68	7.93	-1.66	-0.74	6.54
4	14N	Nitrogen	n/a	8.03	5.98	2.59	-1.5	1.51
		avg. (SE)	*0.76*	*4.98 (2.87)*	*6.87 (0.55)*	*0.54 (0.89)*		
1	4P	Phosphorus	0.89 ± 0.03	4.56	1.37	14.49	-4.13	8.12
2	8P	Phosphorus	0.83 ± 0.02	5.83	3.47	10.6	-3.35	9.36
3	12P	Phosphorus	n/a	4.97	7.22	16.61	-5.29	6.87
4	15P	Phosphorus	0.51 ± 0.03	1.74	3.74	16.23	-2.41	11.28
		avg. (SE)	*0.74*	*4.28 (0.89)*	*3.95 (1.21)*	*14.48 (1.37)*		
1	3NP	N + P	0.41 ± 0.01	6.05	5.15	2.06	-0.82	13.66
2	7NP	N + P	0.59 ± 0.01	14.85	5.08	2.67	-1.11	11.5
3	11NP	N + P	0.42 ± 0.04	-2.3	4.47	0.36	-0.85	7.46
4	16NP	N + P	0.41 ± 0.03	-2.93	4.29	1.52	-1.43	9.47
		avg. (SE)	*0.46*	*3.92 (4.18)*	*4.75 (0.22)*	*1.65 (0.49)*		

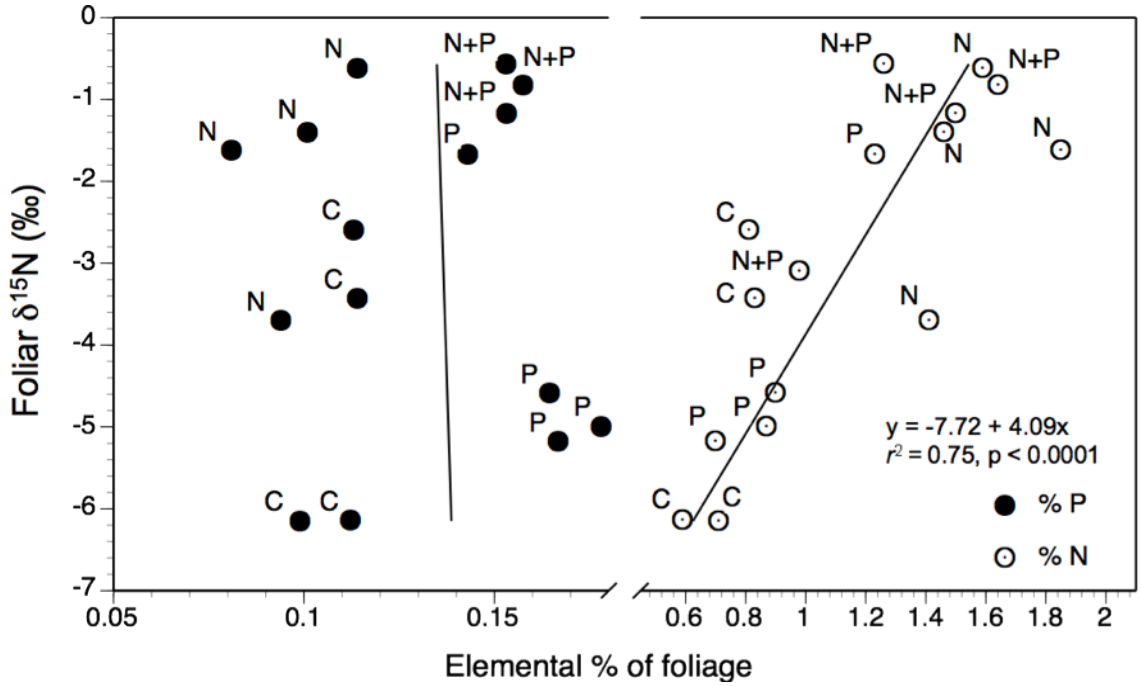

Figure 4-1. Foliar $\delta^{15}N$ values from black spruce trees were strongly correlated with %N across fertilization treatments ($\delta^{15}N = -7.72 + 4.09 \times$ %N, $R^2 = 0.75$, $P < 0.001$) but not %P. Each point represents the mean of three or four trees from each of the 16 fertilized plots.

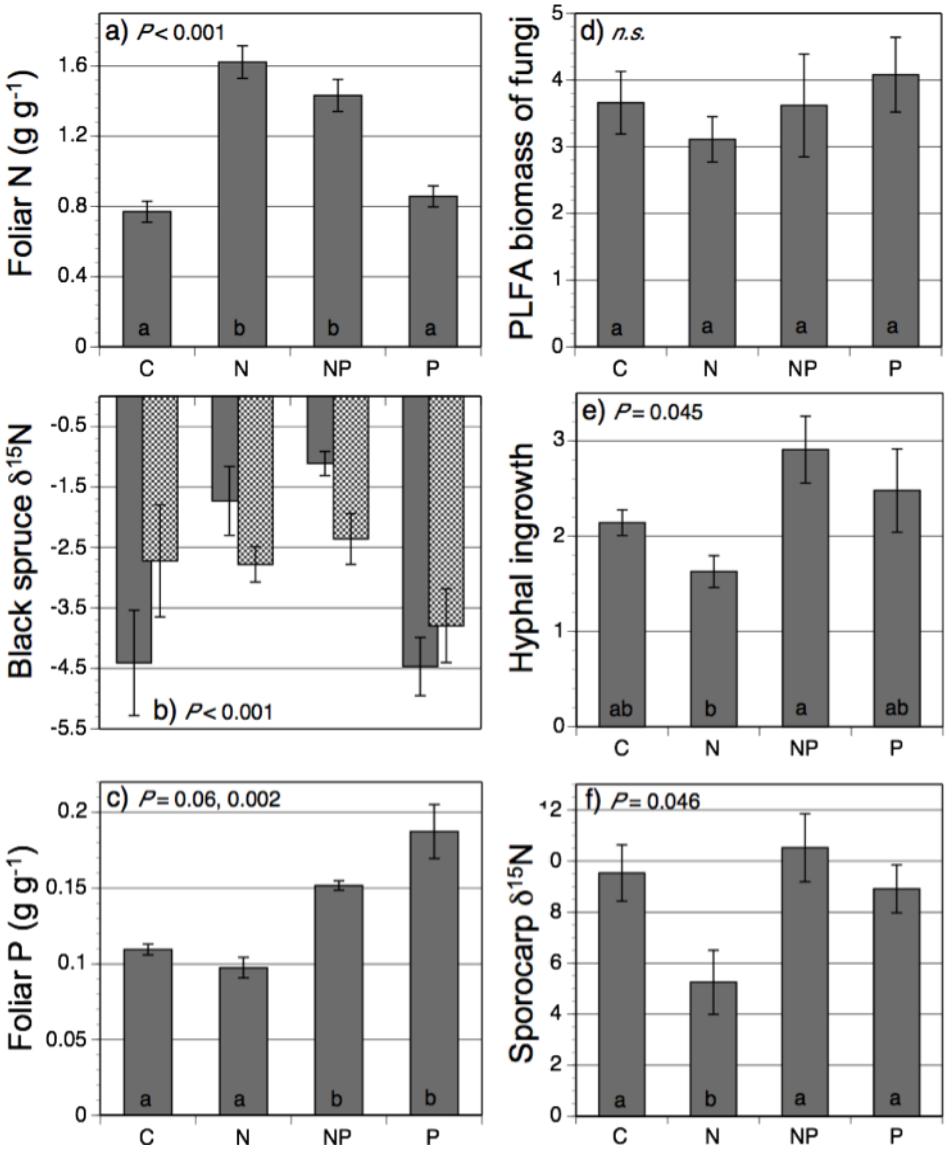

Figure 4-2. Black spruce and fungal response ± *SE* to five years of fertilization with nitrogen (N), phosphorus (P), both (NP), or none (C). Different letters indicate differences either relative to the control (Dunnett's ANOVA with blocking effect) in panels' a-d, or Tukey's HSD with blocking effect panels' e-f. (A) foliar N concentration of mature black spruce. (B) Black spruce needle (grey bars) and root (checkered bars) $\delta^{15}N$ values. (C) Black spruce needle P concentration. (D) Organic soil content of the specific phospholipid fatty acid (PLFA) 18:2ω6,9 regarded as a metric of fungal biomass. (E) Hyphal ingrowth measured in sand filled mesh bags incubated for the 2007 growing season. (F) Ectomycorrhizal sporocarps opportunistically collected from the plots during 2005-2008.

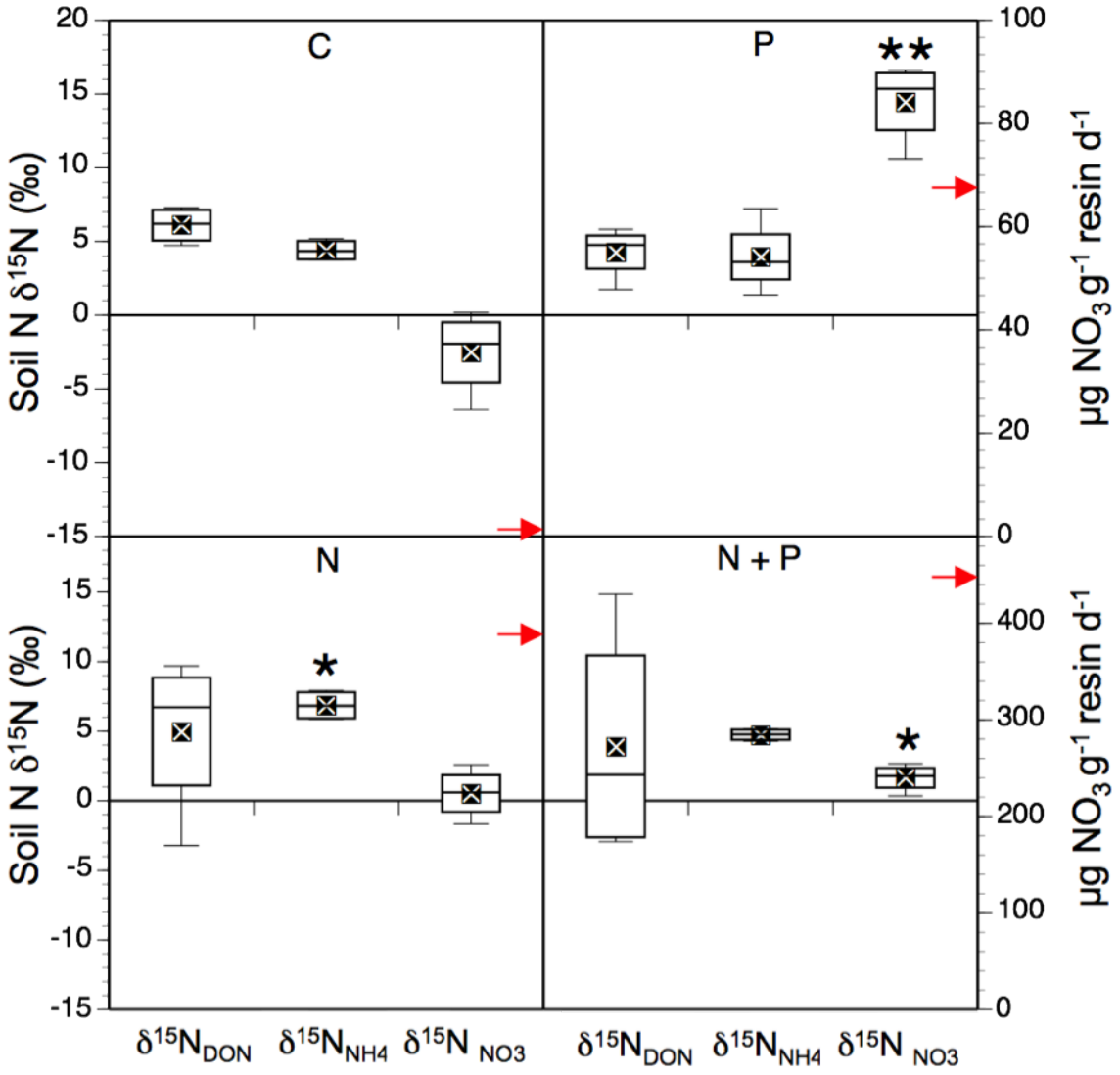

Figure 4-3. Responses of soil N $\delta^{15}N$ values to five years of fertilization with ammonium nitrate (N), orthophosphate (P), both (N + P), or none (control). Double asterisks indicates differences from control at $\alpha = 0.05$, single asterisk at $\alpha = 0.10$. The $\delta^{15}N$ value of resin extractable NH_4^+ was enriched in the N treatment relative to the control ($P = 0.07$, Dunnett's test) and in the N treatment relative to the P treatments ($P < 0.1$, Tukey's HSD). The $\delta^{15}N$ value of resin extractable NO_3^- was enriched in P relative to all treatments ($P < 0.001$, Tukey's HSD) and the N+P treatment relative to the control ($P = 0.07$, Dunnett's test). Red arrows on right axis of figures indicate the average resin exchangeable [NO_3^-] for each treatment as a corollary to the spike in $\delta^{15}N_{NO3}$ enrichment. Note that the scaling of NO_3^- concentration was adjusted between treatments with and without N fertilization.

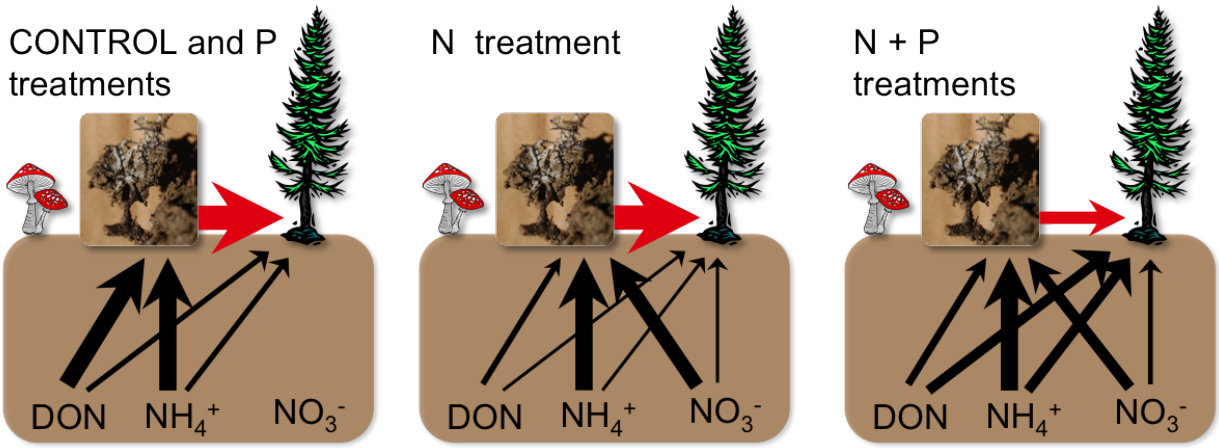

Figure 4-4. Model of the patterns of N fluxes across our treatment types as informed by mass balance mixing models. The Control and Phosphorus (P) treatments illustrate the high proportional flux of N through ECM hyphae (photo inset) to black spruce (red arrow), a high dependency on DON, and a lack of NO_3^- use. The N treatment in the middle illustrates a more complex pattern where NH_4^+ and NO_3^- fertilizer are seen to supplant reliance on DON uptake yet reliance on ECM derived N remains unchanged. The N + P treatment on the right illustrates the most complex pattern of nutrient uptake with similar reliance upon N fertilizer but with a reduced reliance on ECM fungi.

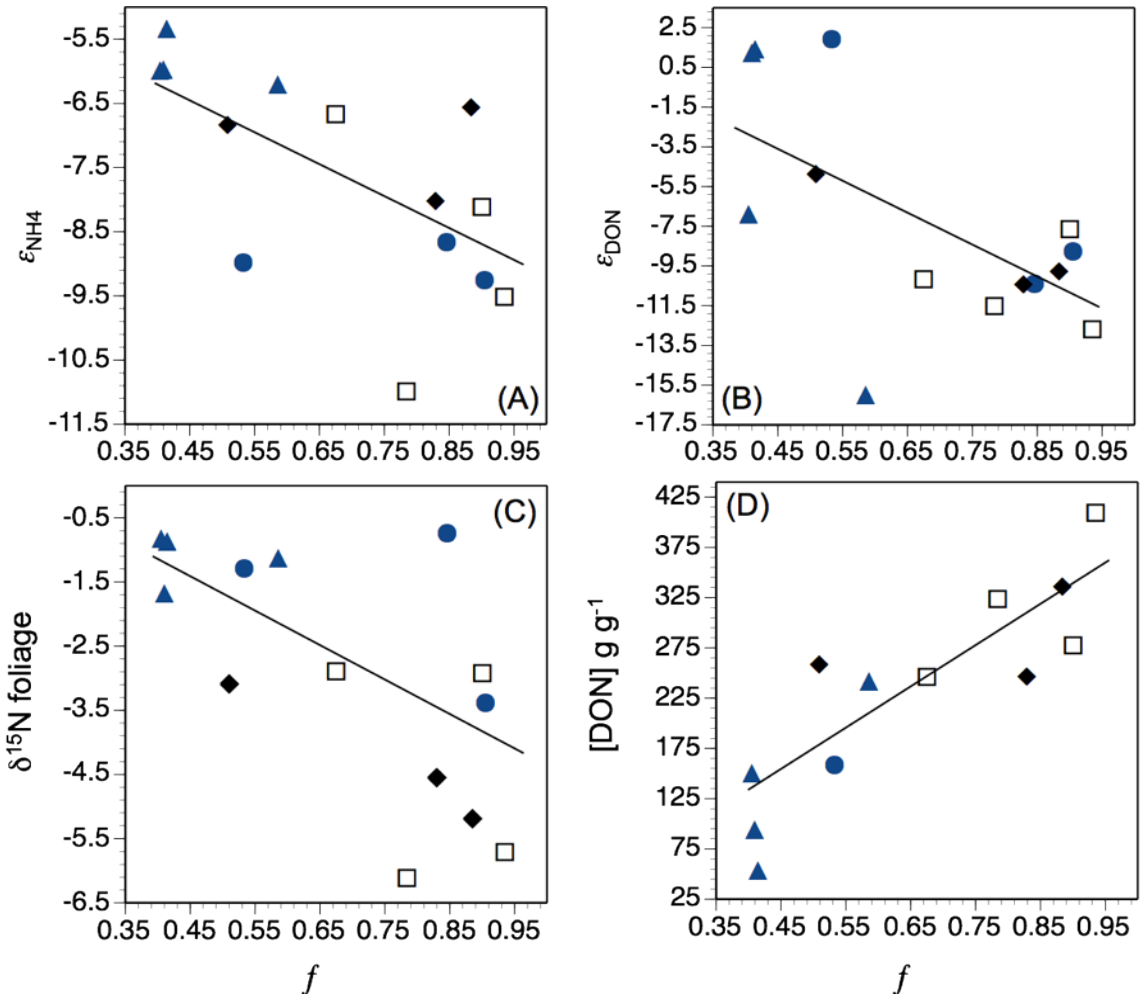

Figure 4-5. Correlations between black spruce proportional dependence on ECM derived N (f) and other isotopic components in experimentally fertilized black spruce forest in central Alaska. (A) An NH_4^+ based enrichment factor ($\varepsilon = \delta^{15}N_{foliar} - {}_{NH4}$), $R^2 = 0.43$. (B) A DON based enrichment factor ($\varepsilon = \delta^{15}N_{foliar} - \delta^{15}N_{DON}$), $R^2 = 0.41$. (C) Black spruce foliar δ^{15}N, $R^2 = 0.43$. (D) Salt extracted soil [DON], $R^2 = 0.75$. Open square = Control, blue circle = N, blue triangle = N+P, black diamond = P treatment.

APPENDIX A
COLLECTOR BASED MISSCLASSIFICATIONS OF FUNGI

A-2 Potential collector based misclassifications based on multivariate discriminant analysis of site normalized fungal isotope values ($\delta^{13}C$, $\delta^{15}N$). Type = collector based classification as mycorrhizal (m) or saprotrophic (s), ECM_P = percent probability of sporocarp being ectomycorrhizal or saprotrophic (SAP_P), * weak, ** = moderate, *** = strong support for assigned category.

Author	Species	Site	Type	ECM_P	SAP_P	
Kohzu et al. 1999	*Russula sp.*	Asihu	m	37	63	*
	Laccaria sp.	Asihu	m	17	83	**
	Trametes versicolor	Asihu	s	69	31	*
	Agaricus subrutilescens	Asihu	s	100	0	***
	Coprinus sp.	Asihu	s	84	16	**
	Pholiota lenta	Ashiu	s	69	31	*
	Pleurotus ostreatus	Chiba	s	96	4	***
	Amanita abrupta	Kyoto	m	49	51	*
	Amanita esculenta	Kyoto	m	37	63	*
	Amanita pantherina	Kyoto	m	36	64	*
	Boletus pseudocalopus	Kyoto	m	28	72	*
	Boletus pseudocalopus	Kyoto	m	37	63	*
	Boletaceae sp.	Kyoto	m	49.7	50.3	*
	Cortinarius sp.	Kyoto	m	4	96	***
	Entoloma clypeatum	Kyoto	m	11	89	**
	Lactarius chrysorrheus	Kyoto	m	43	57	*
	Lactarius chrysorrheus	Kyoto	m	36	64	*
	Lentinula edodes	Kyoto	s	71	29	*
	Psathyrella sp.	Kyoto	s	55	45	*
	Marasmius maximus	Kyoto	s	97	3	***
	Mycena pura	Kyoto	s	73	27	*
	Mycena sp.	Kyoto	s	92	8	***
	Tylopilus sp.	Lambir	m	6	94	***
	Tylopilus sp.	Lambir	m	42	58	*
	Ganoderma australe	Lambir	s	84	16	**
	Microporus vernicipes	Lambir	s	91	9	***
	Trametes versicolor	Lambir	s	58	42	*
	Mycoleptodonoides aitchisonii	Oodai	s	60	40	*
	Armillariella mellea	Oodai	s	65	35	*
	Trametes sp.	Shirahama	s	67	32	*
Hobbie et al. 2001	*Psathyrella sp. #2*	Woods Creek	s	80	20	**
	Psathyrella sp. #1	Woods Creek	s	53	47	*
	Galerina heterocystis	Woods Creek	s	94	6	***
Henn & Chapela 2001	*N/A*	California pine forest	s	60	40	*
Taylor et al. 2003	*Chalciporus piperatus*	Aheden	m	40	60	*
	Russula betularum	Stadsskogen	m	36	64	*
	Russula vinosa	Stadsskogen	m	49.5	50.5	*

	Gymnopilus junonius	Stadsskogen	s	56	44	*
Trudell et al. 2004	*Cortinarius variosimilis*	Deer Park Rd	m	2	98	***
	Cystoderma amianthinum	Deer Park Rd	s	69	31	*
	Cystoderma granulosum	Deer Park Rd	s	59	41	*
	Pseudoplectania melaena	Deer Park Rd	s	62	38	*
	Mycena clavicularis	Hoh rainforest	s	61	39	*
	Pseudoplectania melaena	Hoh rainforest	s	64	36	*
Hart et al. 2006	*Inocybe geophylla*	Lamar Haines	m	39	61	*
	Agaricus silvicola	Lamar Haines	s	65	35	*
	Gymnopilus bellulus	Lamar Haines	s	94	6	***
	Pholiota squarrosa	Lamar Haines	s	96	4	***
	Pluteus lutescens	Lamar Haines	s	82	17	**
	Hygrophorus camarophyllus	Snowbowl	m	39	61	*
	Inocybe lacera	Snowbowl	m	19	81	**
	Pholiota squarrosa	Snowbowl	s	91	9	***
Zeller et al. 2007	*Lactarius chrysorrheus*	Breuil, France	m	44	56	*
	Leotia lubrica	Breuil, France	s	99	1	***
	Amanita citrina	Spruce plantation	m	47	53	*
	Clitopilus prunulus	Spruce plantation	s	62	38	*
	Hypholoma fasciculare	Spruce plantation	s	66	34	*
	Micromphale perforans	Spruce plantation	s	63	37	*
Mayor et al. this study	*Boletellus exiguus*	Guyana	m	41	59	*
	Cantherellus pleurotoides	Guyana	m	7	93	***
	Cantherellus pleurotoides	Guyana	m	21	79	**
	Cantherellus pleurotoides	Guyana	m	23	77	**
	Russula sp.	Guyana	m	13	87	**
	Tylopilus potamogeton var. irengensis	Guyana	m	22	78	**
	Perenniporia inflexibilis	Guyana	s	57	43	*
	Stipitochaete damaecornis	Guyana	s	58	42	*
	Xylaria sp.	Guyana	s	71	29	*
	“Collybia” aff. laccata	Guyana	s	68	32	*

APPENDIX B
CLASSIFICATION OF FUNGI WITH UNKNOWN ECOLOGY

B-2 Classification of fungi with unknown (unk) ecological roles based on multivariate discriminant analyses of site normalized fungal isotope values ($\delta^{13}C$, $\delta^{15}N$). "ECM_P" = percent probability of sporocarp being ectomycorrhizal or saprotrophic (SAP_P), * weak, ** = moderate, *** = strong support for assigned category. Descriptions of the sites can be found within the referenced articles detailed in the main text.

Author	Species	Site	Type	ECM_P	SAP_P	
Hobbie et al. 2001	*Helvella lacunosa*	Woods Creek	unk	89	11	**
	Otidia aff. concinna	Woods Creek	unk	94	6	***
	Clavulina cf. cristata	Woods Creek	unk	98	2	***
	Ramaria	Woods Creek	unk	93	7	***
	Helvella crispa	Woods Creek	unk	97	3	***
	Otidea onotica	Woods Creek	unk	99	1	***
	Helvella sp.	Woods Creek	unk	7	93	***
	Ramaria sp.	Woods Creek	unk	100	0	***
	Clavulina cristata	Woods Creek	unk	99	1	***
	Clavulina rugosa	Woods Creek	unk	99	1	***
Trudell et al. 2004	*Phylloporus rhodoxanthus*	Deer Park Rd	unk	88	12	**
	Tricholoma vaccinum	Deer Park Rd	unk	99.5	0.5	***
	Bondarzewia mesenterica	Hoh rainforest	unk	36	64	*
	Entoloma nitidum	Hoh rainforest	unk	81	19	**
	Phylloporus rhodoxanthus	Hoh rainforest	unk	81	19	**
Hart et al. 2006	*Lycoperdon perlatum*	Lamar Haines	unk	1	99	***
	Bovista spp.	Snowbowl	unk	47	53	*
	Lycoperdon perlatum	Snowbowl	unk	7	93	***
	Lycoperdon pyriforme	Snowbowl	unk	0	100	***
Clemmensen et al. 2006	*Fayodia bisphaerigerella*	Tussock tundra	unk	71	29	*
Mayor et al. this study	*Agaricus sp.*	Guyana	unk	99	1	***
	Coltriciella navispora	Guyana	unk	97	3	***
	Coltriciella navispora	Guyana	unk	93	7	***
	Coltriciella navispora	Guyana	unk	93	7	***
	Coltriciella oblectabillis	Guyana	unk	99	1	***
	Gymnopilus sp.	Guyana	unk	50.2	49.8	*
	Tremellodendron ocreatum	Guyana	unk	91	9	***

APPENDIX C
ISOTOPE MASS BALANCE MIXING RESULTS

C-3 Supplementary table of mass balance mixing model results on a plot-by-plot basis. See Methods for a definition of terms. $\delta^{15}N$ values were measured, green values are the average of all plots, red is the highest value recorded among all plots which was substituted to achieve mass balance where indicated.

PLOT	PARAMETERS					
	f_{NH4}	f_{NO3}	T_r	f	$\delta^{15}N_{fungi}$	avg f
TKN-2	0.1		.23-.32	0.68	11.56‰	0.60
	0.3		.27-.35	0.6		
	0.5		.31-.38	0.53		
TKN-4	0.5		.46-.54	1	6.51‰	1.00
TKN-12	0.0		.70-.74	0.91	3.79‰	0.91
TKN-16	1		.19-.3	0.97	11‰	0.97
TKN-21	1		.2-.3	0.99	11‰	0.99
TKN-22	1		.3-.38	0.95	11‰	0.95
TKN-30	1		.42-.49	1	8.5‰	1.00
TKN-32	0.7		.45-.53	0.98	6.51‰	0.98
TKN-34	0.5	0.4	.54-.6	0.81	6.51‰	0.84
	0.4	0.4	.54-.59	0.84		
	0.3	0.4	.53-.59	0.87		
TKN-39	1		.34-.41	0.97	11‰	0.97
TKN-40	0.8		.52-.58	0.96	6.51‰	0.89
	0.9		.54-.59	0.89		
	1		.55-.6	0.83		
TKN-43	0.1		.18-.29	0.92	11‰	0.91
	0.3		.19-.3	0.91		
	0.5		.21-.31	0.89		
TKN-51	0.5		.24-.33	0.93	11‰	0.93
TKN-54	0.1		.35-.43	0.89	8.69‰	0.92
	0.3		.34-.43	0.9		
	0.5		.34-.43	0.91		
	0.4	0.1	.33-.42	0.93		
	0.3	0.2	.32-.41	0.95		
TKN-114	0.3		.15-.26	0.98	11‰	0.96
	0.5		.18-.29	0.94		
TKN-120	0.3	0.2	.47-.54	1	6.51‰	0.91
	0.2	0.3	.51-.57	0.87		
	0.3	0.3	.51-.57	0.87		
TKN-127	0.6		.54-.59	0.96	6.51‰	0.83
	0.7		.57-.62	0.82		
	0.8		.59-.64	0.7		
TKN-131	0.1		.23-.33	0.91	11‰	0.93
	0.3		.23-.32	0.93		

	0.5		.22-.32	0.94			
TKN-133	0.5		.13-.25	0.99	11‰	0.99	
TKN-207	1		.08-.21	1	11‰	0.99	
TKN-210	0.8		.56-.61	1	5.8‰	0.88	
	0.9		.58-.63	0.89			
	1		.6-.65	0.76			
TKN-213	1		.32-.4	1	11‰	1.00	
TKN-214	0.8		.26-.35	0.94	11‰	0.87	
	1		.34-.41	0.79			
TKN-222	0.1		.24-.35	0.99	9.09‰	0.82	
	0.3		.25-.36	0.96			
	0.5		.27-.37	0.5			
TKN-223	0.5		.43-.52	1	6.51‰	0.92	
	0.7		.5-.57	0.83			
TKN-225	0.1		.28-.36	0.77	11‰	0.67	
	0.3		.29-.37	0.74			
	0.5		.31-.38	0.5			
TKN-235	1		.32-.4	1	11‰	1.00	
TKN-237	0.1		.5-.56	0.92	6.51‰	0.94	
	0.2	0.1	.5-.57	0.93			
	0.3	0.1	.49-.56	0.98			
jrm2	0.3		.08-.21	0.93	11‰	0.89	
	0.5		.15-.26	0.84			
jsn	0.1		.47-.54	0.82	6.51‰	0.65	
	0.3		.51-.58	0.65			
	0.5		.55-.61	0.47			
DJ	0.1		.17-.29	0.93	9.79‰	0.89	
	0.3		.2-.31	0.89			
	0.5		.22-.33	0.86			
					Average	0.90	±0.02

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BIOGRAPHICAL SKETCH

Jordan Mayor grew up in northern Virginia and became interested in biology through Natural History and National Geographic magazines and during an advanced placement biology course in high school. These interests were supplemented through early explorations of the nearby woods with his mother and later alone in the neighboring Appalachian Mountains. He began his college career at George Mason University in 1994 at the age of 17 where he took a variety of formative courses ranging from Mushrooms, Molds, & Molecules taught by Albert Torzilli to Human Ecology from Mary Catherine Bateson. After soon exhausting the available biology courses he transferred to Virginia Polytechnic Institute & State University as a biology major. There he worked on undergraduate research projects related to the mycorrhization of pine trees under the late and great Dr. Orson K. Miller Jr. It is here he also had his first opportunities to conduct conservation biology field work chasing around red cockaded woodpeckers through chigger-infested long leaf pine woods of North Carolina. After completing his B.S. in Biology in 1999, he moved to Northern California for more naturalizing. He worked a variety of positions throughout Northern California and Oregon mountains including as: a stream restoration intern to create salmon habitat; a wildlife biologist hooting for spotted owls late into the night; a Forest Service technician crawling about looking for rare mollusks, salamanders, and plants; and, as an independent subcontractor conducting rare plant and fungi surveys. After four years of sleeping under the stars, swimming in streams, tromping through high desserts, and drying gear in middle-of-nowhere motels, he returned to academia as a Master's student with Terry Henkel at Humboldt State University (HSU) in 2003. At HSU he conducted his research in tropical rainforests of Guyana focused on the influence of

ectomycorrhizal fungi on leaf litter decomposition. This work involved participating and leading four expeditions to the remote Pakaraima Mountains where he lived and worked among Patamona Amerindians under luxurious tarp and hammock accommodations. It is also here where he developed an inordinate fondness for dried beans. After two years he finished up and moved to FL to work on this PhD at University of Florida with Ted Schuur in 2005. After a brief tropical stint collecting in Guyana and assisting a postdoc in Colombia, he quickly realized the benefit that refrigeration and laboratory facilities could provide to the interpretability of his soil N extractions. He also began to believe that it is questions that should drive science, not the system or techniques. These beliefs, combined with logistical concerns, led him to work in Alaska, where the promise of laboratories and preexisting data overpowered his love of tropical forests. He does not regret that decision because an ecosystem ecologist should truly have a global perspective.

Upon submission of this dissertation he will return to tropical forest but with a question driven approach backed by ample methodological experience. He will conduct similar fundamental research based on understanding the underlying controls and utility of $\delta^{15}N$ measurements throughout Panamanian rainforest thanks to a generous National Science Foundation International Research Fellowship. This work will be a natural extension of his PhD work and will permit him to address multiple competing hypotheses in a diverse, dynamic, and N-rich system.